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VARIABILITY OF *BACILLUS SUBTILIS* IN RELATION TO ANTIBIOTIC PRODUCTION

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THE UNIVERSITY OF ALBERTA

VARIABILITY OF *BACILLUS SUBTILIS* IN RELATION TO ANTIBIOTIC PRODUCTION

A DISSERTATION
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ABSTRACT

Fifty-one strains of Bacillus subtilis, isolated from various materials, were studied for variability in antibiotic production. The isolates varied greatly in their antibiotic properties. Some proved to be inactive, but the majority were either moderately or strongly active towards the test bacteria used.

When the antibacterial activities of the strains were compared with that of B. subtilis A32, many of the isolates were found to be considerably less active while others exhibited degrees of activity equal to that of the A32 strain. Differences in antibiotic activity believed to be related to differences in the types of antibiotic produced were exhibited when the antibacterial spectra were compared with the spectrum of A32.

High antibiotic activity was found to be closely associated with the lactose fermenting ability of the isolates studied. Other physiological and morphological characteristics were less closely associated with the antibiotic properties of the strains.

Paper partition chromatography gave negative results when solvent systems employing water as the stationary phase were used. Reversed phase paper chromatography, however, gave more promising results.

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VARIABILITY OF *BACILLUS SUBTILIS* IN RELATION TO ANTIBIOTIC PRODUCTION

R. L. Millar

INTRODUCTION

Antibiotics have been derived from fungi, actinomycetes and bacteria. Not only different species of each, but even strains of a single species have been shown to differ in the production of antibiotics. The variable nature of this property may be evidenced in the amount of a particular antibiotic produced or in the type or types of antibiotic produced.

Among the fungi exhibiting this property are species of Penicillium. These have been shown to vary greatly in their ability to produce the antibiotic penicillin. Winsten and Spark (1) have shown that *Penicillium chrysogenum* Thom Q-176 may produce as many as eight different penicillin types.

Streptomyces griseus (Krainsky) *comb. nov.* is representative of the actinomycetes, which have yielded a number of antibiotics possessing different biological and chemical properties. Schatz *et al* (2) was the first to report the production of the substance, streptomycin, by this

organism. Grisein (3), actidione (4) and another factor (5) have since been obtained as additional products from it.

Bacillus polymyxa (Prazmowski) Migula and Bacillus subtilis Cohn emend Prazmowski are bacteria which are exceptionally good sources of antibiotic substances. Porter et al. (6), reported that there appeared to be important differences among 65 isolates of B. polymyxa in respect to their antibiotic production. Varying degrees of effectiveness of the isolates were demonstrated against the test organism used. Several substances have been isolated from B. polymyxa which are very closely related chemically and practically indistinguishable biologically. The name polymyxin has been given to these substances as a group, and the individual compounds have been distinguished by the letters A, B, C, and D.

Bacillus subtilis has yielded a greater number of antibiotic entities than has any other bacterium. Marked variability in the chemical nature of these has been clearly shown. The first to be described was subtilin, a polypeptide isolated by Jansen and Hirschmann (7). This was found to occur within the cells of the organism. Bacitracin (8), another antibiotic produced by B. subtilis, differs from subtilin by accumulating in the culture liquor, free from the cells. A third agent of B. subtilis, bacillin, has been described by Foster and Woodruff (9). Still other products of this organism are subtilysine (10), endosubt-

ilysin (11), subtiline (12), bacilipin A and B, bacilysin (13), eumycin (14), subtenolin (15), and licheniformin (16). There are in addition a number of factors which have been reported but which have not as yet been named.

PREVIOUS STUDIES ON THE ANTIBIOTIC PROPERTIES OF *BACILLUS SUBTILIS* AT THE UNIVERSITY OF ALBERTA

The strain of *Bacillus subtilis*, referred to here as A32, was isolated by Jackson (17) and later identified by Dr. N. R. Smith of the United States Department of Agriculture. On the basis of its non-phytotoxicity and its antagonism to *Xanthomonas translucens f. sp. hordei-avenae* (S. J. and R.) Dowson emend Hagb., the A32 strain was selected by J. J. Goodman (18) for detailed study.

Goodman's investigation dealt firstly, with the isolation and purification of an antibiotic from this organism and with factors affecting its production and secondly, with the use of this antibiotic in the control of the bacterial pathogen *Xanthomonas translucens* which causes Bacterial Blight of barley.

Further studies on the purification of the active substance produced by A32 were made in co-operation with the Chemistry Department of the University. Gordon (19), under the supervision of Dr. R. B. Sandin developed a method by

which the antibiotic could be obtained in a partially purified form.

In the investigations reported here, A32 has been used as a standard to which the antibiotic properties of a number of other strains of B. subtilis have been compared.

OBJECTS OF THE INVESTIGATION

The main objects of the investigations reported here were as follows:

1. To determine if the strain of Bacillus subtilis referred to as A32 can be commonly isolated from various local sources and in particular from seed and soil.
2. To study the ability of local strains of Bacillus subtilis to produce antibiotically active material and to determine if the strains which produce appreciable amounts differ from the A32 strain in either the quantity or in the type of antibiotic produced.
3. To determine if the ability of strains of Bacillus subtilis to produce antibiotically active material is associated with any physiological or morphological characteristic of the strain.

SOURCES OF THE ISOLATES

Introduction

Bacillus subtilis, one of the spore-forming bacteria, is widely distributed in nature and has been isolated from a number of sources. Soil and decomposing organic matter have been reported (20) as natural habitats of this organism. However, plant vegetation and the air have proved to be other fruitful sources.

The subtenolin-producing strain was obtained by Hirschorn *et al.* (15) from agar plates that had been seeded with *Escherichia coli* (Migula) Castellani and Chalmers and *Staphylococcus aureus* Rosenbach, respectively, and inoculated with material from dusty surfaces in the laboratory. Similarly, Johnson and Burdon (14) isolated the strain which yields eumycin by exposing agar plates to contamination with dust. Johnson *et al.* (8) isolated the bacitracin strain from cultures of contaminated tissue removed at operation from a compound fracture of the tibia. A strain, isolated from a contaminated culture of *Mycobacterium tuberculosis var. hominis* (Schroeter) Lehmann and Neumann was found by Newton (13) to produce bacilipin A, bacilipin B and bacilysin. The strain which yields bacillin (9) and the A32 strain were obtained as isolates from soil.

In the present investigations a number of mater-

ials were tested for the presence of Bacillus subtilis. Fifteen samples of wheat seed and fourteen samples of soil obtained from quite widely separated localities in Alberta were tested particularly for the presence of those organisms exhibiting a likeness in growth characteristics to those of the A32 strain. Materials tested for the presence of this and other strains of B. subtilis were seed (wheat, oats, barley, peas, lupine, sugar-beet, sunflower), wheat flour, ground pea material, oat glumes and air.

Isolation of B. subtilis from Different Sources

Isolation from wheat seed following hot-water treatment

During the investigations on the isolation of B. subtilis from various materials, wheat seed which had been hot-water treated was tested as a potential source. Eight isolates resembling the A32 strain in their growth characteristics were obtained. This suggested that the A32 strain might be a common contaminant of hot-water treated seed. To test this possibility, the following method was employed. As reported here, the method represents a modification of the hot-water method developed as a control for the disease Loose Smut of wheat.

The seed used consisted of samples of wheat obtained from fifteen locations in northern Alberta. Duplicate lots of seed from each of the samples were tested in two plates, each containing seven or eight seeds.

tain were tested for the presence of Penicillin amidase.
Strong evidence of enzyme exist was furnished by the
result obtained from quite widely separated localities in
Illinois were tested particularly for the presence of those
organisms exhibiting a tendency to secrete this enzyme
to those of the ABC variety. Materials tested for the pres-
ence of this new enzyme activity of P. multilis were sand
(wheat, corn, barley, peas, lupine, sugar-beet, soybean,
wheat flour, grass (pea pasture), oat straws and air.

Isolation of P. multilis from Different Sources

Isolation from wheat seed following out-door treatment

In P. multilis from various materials, wheat seed which had
been out-door between rye listed as a potential source.
Eight isolates resembling the ABC strain in their growth
characteristics were obtained. This suggested that the ABC
strain might be a common contaminant of out-door treated
seed. In case this possibility, the following experiment
was designed. In selected jars, the culture was of one
isolation of the pure-culture isolates described as a control
for the disease index four to eight.

The same two isolations of multilis it would di-
fferent from Illinois, however in morphological (Fig.). Morpho-
logic type of each from each of the strains were tested in
the dishes, each containing seven or eight seeds.

Small glass cylinders containing 5 ml. of distilled water were bunged and sterilized. Lots of seeds of each of the various samples were added to a number of the glass cylinders and sterile, perforated glass-beads were added, as checks, to the remainder.

The seeds were allowed to soak for 4 hours at a temperature of 15° - 20°C. At the end of this pre-soak period, the cylinders containing the seeds were transferred to a hot-water bath (49°C) for 1 minute. Following this pre-heating period they were transferred to a second bath where they were kept for 10 minutes at a temperature of 51° - 53°C. The cylinders were then placed in cold water to cool their contents.

The seeds and the bead-checks were transferred from the cylinders to potato-dextrose agar plates in an inoculation chamber which was washed down prior to the treatment with 1:1000 mercuric chloride solution and then irradiated with ultra-violet light for 1 hour. One-ml. of the water suspension remaining in the cylinders, following removal of the seeds and the bead checks, was mixed with about 15 ml. of potato-dextrose agar in a Petri plate. The agar was cooled to about 46°C before it was mixed with the water suspensions. As soon as the agar had hardened all of the plates were placed in a 30°C incubator.

None of the organisms that appeared in the plates containing the seeds resembled A32 in their growth characteristics when they were grown on potato-dextrose agar slants.

A similar result was obtained from those plates prepared from the water suspensions. No growth was apparent in any of the check plates.

These results indicate that the A32 strain does not commonly occur as a contaminant of hot-water treated seed. The eight isolates previously mentioned, were obtained from seeds which had been treated without adequate control being taken against contamination from the air, or from equipment used in the test. The fact that such isolates were not obtained when the test was properly controlled against contamination indicates the necessity of suitable precautions being taken whenever investigations of this nature are performed.

Isolation from the Soil

The A32 strain, as reported by Goodman (18), was isolated from Edmonton black soil. In order to determine the prevalence of this strain in soil, samples of soil from widely separated localities were collected and subsequently tested by the following methods.

Each sample was thoroughly mixed within the sample tube with a sterile needle. The needle was then flamed, cooled by wetting with 0.85 percent sodium chloride solution and plunged into the soil sample. The soil particles which adhered to the needle when it was withdrawn were transferred to 10 ml. of sterile saline solution. This process was repeated with each soil sample to give duplicate stock suspensions.

A similar result was obtained from three Cheyenne subsoil
from the water emplacements. In growth was apparent in any
of the check plants.

These results indicate that the 32 metals that
not commonly occur as a contaminant of soil-water treated
sand. The eight isolates positively monitored, were obtain-
ed from seeds which had been treated without subsequent sterili-
zation. Using taken aqueous suspensions from the air, or from
equipment used in the test. The test dust soil isolates
were not obtained when the test was properly controlled
against contamination including the necessity of autoclav-
ing tubes and pipettes. Preservation of isolations of fungi
cultures are performed.

Isolation from the soil

The 130 strain, as reported by Downes (19), was
isolated from Edmonton clay soil. In order to determine
the prevalence of this strain in soil, samples of soil from
widely separated localities were collected and examined for
the presence of the following metals:

Each sample was moistened, mixed with the equiva-
lent of a specific metal. The results are shown below.
Counts of melting pits (.05 percent acting colonies solution
and diluted into one soil sample. The soil particles which
adhered to the metals when it was withdrawn were transferred
to 10 ml. of sterile saline solution. This process was
repeated with each soil sample to give duplicate inocu-
lations.

The stock suspensions were treated as follows:

(a) The suspensions were left standing for one hour and then shaken for 1-2 minutes to free the bacteria from the soil particles;

(b) Following the above procedure the suspensions were pasteurized (80°C for 10 minutes), cooled in running tap water, and kept overnight.

The pasteurized and the non-pasteurized stock suspensions were then tested by the following methods for the presence of the A32 strain:

(a) One loopful of each suspension was transferred to sterile potato slices and incubated at 40°C for 48 hours;

(b) One-ml. lots of the suspensions were transferred to 10 ml. lots of asparagine broth and incubated at 30°C for 4 days. Following the incubation period, streak plates of each broth culture were prepared on potato-dextrose agar after which the plates were incubated until definite growth had been made;

(c) One-ml. lots of the suspensions were serially diluted to 1:1000 dilutions. Each of the stock suspensions and the 1:1000 dilutions were then plated and incubated at 30°C for 5 days.

To detect contaminants from sources other than those under test the following checks were included:

(a) Sterilized samples of Edmonton black soil;

(b) Sterilized samples of soil to which had been

added one loopful of a suspension of A32;

(c) Suspensions of A32 alone.

The checks were treated in exactly the same manner as the collected soil samples with duplicate preparations made in each case.

Aside from the checks the test yielded only one colony of an organism whose growth characteristics on potato-dextrose agar resembled those of A32. This isolate was obtained from a 1:1,000 dilution plate of a non-pasteurized soil suspension. Except for this single isolate, the presence of the A32 strain in the soil was demonstrable only in the sterilized samples to which it had been added. A32 was also recovered from those plates and potato slices prepared from the A32 suspensions.

The results obtained by this test were in agreement with a previous one, not reported here, which failed to yield organisms similar to A32 when Edmonton black soil was investigated as a potential source. The fact that only one isolate was obtained in the test described above suggests that this organism was a contaminant from the air.

Additional evidence in support of these findings was obtained in further studies. In these, suspensions of A32 in dilutions of 1:1,000,000 and 1:100,000,000 were added to test-tubes containing approximately 10 ml. of (a) sterile and (b) non-sterile Edmonton soil. Tubes of soil to which sterile distilled water was added served as checks. All of the tubes were incubated at 30°C for a period of up to two weeks. Each of the soil preparations was tested for the

presence of A32 after intervals of time of 48 hours, 1 week, and 2 weeks had elapsed. This was done in two ways:

(a) By the transfer of soil particles to agar plates;

(b) By preparing agar dilution plates from pasteurized and non-pasteurized suspensions of the various soil preparations.

At no time during the period of the test were organisms of the A32 type recovered from tubes which initially contained non-sterile soil. Similarly A32 was not obtained from sterile soil to which sterile distilled water had been added. Colonies of the A32 type did appear, however, in plates to which soil particles were transferred from the preparations consisting of sterilized soil and the added A32 suspension. They were also produced on agar dilution plates prepared from this soil preparation. The nature of the bacterial growth which appeared in plates prepared from the non-sterile soil preparations was very variable but consisted largely of organisms with a smooth type of growth. This was in direct contrast to the type of growth exhibited by the A32 strain which has been described (18) as being distinctly rough.

Discussion

The growth of A32 on potato-dextrose agar may be described as flat, dull, opaque, and having a rough ground-glass-like topography (18). As organisms resembling A32 in their growth type were not isolated from either pasteurized or non-pasteurized soil, it is concluded that bacteria of

the A32 type do not commonly occur in the soil examined.

Bacillus subtilis occurs as strains whose growth is either rough or smooth in appearance. That A32 could not be recovered from non-sterile soil, to which it had been added, suggests that there may be associated with such soil, some factor or factors which can effect a change in the growth type of this organism. The preponderance of bacterial colonies from the non-sterile soil whose growth was smooth in appearance might be considered as additional evidence in support of this hypothesis. Furthermore, antagonism to A32 by organisms already present in the soil may have had the effect of killing, or at least inhibiting, further growth of cells of this strain.

Isolation from other materials

The following method was used to test for the presence of strains of B. subtilis as contaminants of various kinds of seed.

Seeds were transferred from sterile Petri plates to a 70 percent ethanol solution where they remained for 1-2 minutes. They were then rinsed in sterile distilled water and transferred to potato-dextrose agar plates containing about 20 ml. of agar. The forceps used in making the transfers were flamed between steps. These operations were performed in an inoculating chamber which had been previously washed down with 1:1000 mercuric chloride solution and exposed to ultra-violet irradiation for 1 hour. Sterilized glass beads, which had received the same treatment

as the seeds, served as checks. All plates were incubated at 30°C and were examined daily for the presence of bacterial growth. Only bacterial growth occurring on or immediately around the seeds was transferred to slants for further observation.

Wheat flour, ground pea straw and oat glumes were tested by sprinkling small quantities of them on the surface of agar in plates from a sterile camel's hair brush.

Air-borne isolates were obtained by exposing agar plates to contamination from the air at various locations outside of the laboratory and within the greenhouses.

All of the isolates from these sources were subjected to pasteurization (80°C for 10 minutes) as a preliminary means of selection since B. subtilis is a spore-former and therefore is resistant to this treatment. A list of the isolates, which were later tentatively identified as strains of B. subtilis, the methods used for their isolation and the sources from which they were isolated is given in Table I.

Discussion

A total of 54 strains of B. subtilis were obtained from the various materials tested. Of this number, 8 were isolated as contaminants of hot-water treated seed as previously reported. Subsequent tests, however, indicated that these isolates were from sources other than the hot-water treated seed. The single soil isolate, obtained from a suspension of non-pasteurized soil, on initial transfer to

TABLE I

Strains of B. subtilis isolated from different sources

Culture Designation	Source of isolate	Special treatment employed
V1	wheat (seed)	Hot-water
V2	wheat (seed)	Hot-water
V3	wheat (seed)	Hot-water
V4	wheat (seed)	Hot-water
V5	wheat (seed)	Hot-water
V6	wheat (seed).	Hot-water
V7	wheat (seed)	Hot-water
V8	wheat (seed)	Hot-water
V9	wheat (seed)	Hot-water
V10	wheat (seed)	Hot-water
V11	air	Exposed agar plate
V12	air	Exposed agar plate
V13	oats (glumes)	
V14	peas (straw material)	
V15	Alaska peas (seed)	Alcohol treatment
V16	Tiny peas (seed)	Alcohol treatment
V17	Tiny peas (seed)	Alcohol treatment
V18	air	Exposed agar plate
V19	soil	Soil suspension
V20	air	Exposed agar plate
V21	Tiny peas (seed)	Alcohol treatment
V22	sugar-beets (seed)	Alcohol treatment
V23	air	Exposed agar plate
V24	Alaska peas (seed)	Alcohol treatment
V25	air	Exposed agar plate
V26	air	Exposed agar plate
V27	Marquis wheat (seed)	Alcohol treatment
V28	air	Exposed plate
V29	Marquis wheat (seed)	Alcohol treatment
V30	air	Exposed plate
V31	air	Exposed plate
V32	Victory oats (seed)	Alcohol treatment
V33	Ash seed	
V34	air	Exposed agar plate
V35	air	Exposed agar plate
V36	air	Exposed agar plate
V37	Marquis wheat (seed)	Alcohol treatment
V38	Alaska peas (seed)	Alcohol treatment
V39	air	Exposed agar plate
V40	Tiny peas (seed)	Alcohol treatment
V41	sugar-beet (seed)	Alcohol treatment
V42	Marquis wheat (seed)	Alcohol treatment
V43	Garnet wheat (seed)	Alcohol treatment
V44	Garnet wheat (flour)	
V45	Marquis wheat (seed)	Alcohol treatment
V46	Alton peas (seed)	Alcohol treatment
V47	Alton peas (seed)	Alcohol treatment
V48	Tiny peas (seed)	Alcohol treatment
V49	Victory oats (seed)	Alcohol treatment
V50	Garnet wheat (seed)	
V51	Marquis wheat (seed)	Alcohol treatment
V52	Marquis wheat (seed)	Alcohol treatment
V53	Marquis wheat (seed)	Alcohol treatment
V54	Marquis wheat (seed)	Alcohol treatment

potato-dextrose agar resembled the A32 strain in its growth characteristics. However, on subsequent transfers distinct differences were apparent. These results, under the conditions of the tests, give strong support to the conclusion that the A32 strain does not commonly occur in the soil nor as a contaminant of hot-water treated seed.

Of the remaining isolates, 8 resembled A32 in their growth characteristics. However, only 3 of these were from seed (oat and pea). One was isolated from ground oat glumes, one from pea straw and the other 3 isolates were obtained by exposure of agar plates to contamination from the air. Since one-half of the isolates resembling A32 resulted from contamination of plates during the isolation work it is concluded that the A32 strain is not commonly present on or in the various materials tested. This appears to be particularly true of seed and soil.

IDENTIFICATION OF THE ISOLATES

Introduction

Strains of Bacillus subtilis exhibit marked variations in growth types. Whether the growth is flat, warty, folded, adherent or nonadherent, dry moist, smooth or mucoid depends on the stage of growth (20). The extreme variability exhibited by this organism in its growth characteristics, which might be easily confused with those of other species of the genus, made it apparent that a class-

IDENTIFICATION OF THE ISOLATES

Introduction

Cooking of [illegible] multiple mutual variations in growth types. Neither the broken Ia (IAC, var), Tolaby, Conrad or Ouachita, Ky soil, enough or enough cannula on the stage of growth (3X). The extreme variability exhibited by this organism in its growth characteristics, which might be easily attributed to some other species of the genus, made it expedient that a close-

ification of the isolates as to species was necessary.

The difficulties involved in classifying B. subtilis are reflected in the literature on the subject. The most recent and complete review on the subject has been given by Smith, Gordon and Clark (20). Since there is no universally accepted classification for this organism, all workers reporting on the production of antibiotics by strains of this organism may not have used the same species of aerobic spore-forming bacillus (13).

Gibson and Topping (21) and Gibson (22) have presented one method of classification for B. subtilis. However, in the work reported here the method of classification given by Smith and co-workers (20) has been used for the identification of the isolates.

Methods and Media

The methods used and the media employed were the same as those described by Smith et al. (20) with two exceptions. The following criteria were used in the identification of the isolates:

(a) Pasteurization (80°C for 10 minutes);

(b) Gram's Stain, vegetative cell size and spore size;

(c) Growth on nutrient agar, glucose-nitrate agar and potato-dextrose agar slants;

(d) Growth on potato - potato slices were prepared as described by Fred et al. (23);

(e) Fermentation of carbohydrates (xylose, dextrose, maltose, galactose, lactose and sucrose);

(f) Reduction of nitrates to nitrites - the method recommended in The Manual of Pure Culture Study (24) was used in place of the method outlined by Smith et al. (20);

(g) Voges-Proskauer reaction;

(h) Starch hydrolysis;

(i) Gelatin hydrolysis.

Each of the tests was duplicated for each isolate and in those instances where doubtful results were obtained the tests were repeated. The A32 strain was included with the isolates in all of the tests performed.

On the basis of the identification tests 54 isolates were tentatively identified as strains of B. subtilis. All of these strains are within the limits specified for vegetative cell size and spore size. They are gram-positive and survive pasteurization. They reduce nitrates to nitrites, react positively to the Voges-Proskauer test, and hydrolyze starch and gelatin. Acid without gas is produced by all of the isolates from xylose, dextrose, maltose and sucrose. Acid production from galactose and lactose, however, varies with the strain. Pigment production on potato-dextrose agar varies from creamy-white, to orange, to yellow, to pink. A description of the type of growth on potato-dextrose agar slants (Table II) and on potato slices (Table III) is presented.

TABLE II

Growth characteristics of *B. subtilis* isolates on potato-dextrost agar*

Culture designation	Growth characteristics on potato-dextrose agar
A32	Growth flat, rough, opaque, adherent, cream
V2	Growth as for A32
V3	Growth as for A32
V4	Growth as for A32
V7	Growth as for A32
V8	Growth as for A32
V9	Flat, rough, opaque, slightly adherent, light cream
V10	As for A32
V11	As for A32
V12	As for A32
V13	Flat rough, opaque, adherent, slightly moist, cream
V14	As for A32
V15	Flat to slightly folded, rough opaque, light cream
V16	As for V15
V17	Flat to moderately folded, rough, opaque, non-adherent, light cream
V18	Flat to finely folded, rough, opaque, adherent, light cream
V19	Folded, rough, opaque, non-adherent, moist, light cream
V20	Flat to slightly folded, rough, opaque, adherent, grey
V21	Folded, smooth, opaque, non-adherent, moist dark cream
V22	Finely folded, rough, opaque, slightly moist light cream
V23	As for V22
V24	Folded, smooth, opaque, non-adherent, moist, dark cream
V25	Slightly, folded, rough, opaque, adherent, grey
V26	Folded, rough, opaque, adherent, yellow
V27	Finely folded, rough, opaque, slightly moist, light pink
V28	Flat to folded, rough, opaque, adherent, light cream
V29	Moderately folded, rough, opaque, adherent, light cream
V30	Coarsely folded, rough, opaque, non-adherent, slightly moist orange
V31	Slightly folded, rough, opaque, adherent, slightly moist light cream
V32	As for V26
V33	As for V26
V34	As for V26
V35	As for A32
V36	Flat to finely folded, rough, opaque, adherent, light grey
V37	Finely folded, rough, opaque, adherent cream
V38	As for A32
V39	As for V37
V40	As for V30
V41	Flat to finely folded, rough, opaque, adherent, moist, cream
V42	As for V29
V43	Flat, smooth, opaque, non-adherent, slightly moist, light cream
V44	Flat, rough, opaque, adherent, slightly moist, grey
V45	Flat to finely folded, rough, opaque, adherent, slightly moist, light grey
V46	Slightly folded, smooth, moist, cream
V47	Folded, rough, opaque, moist, dark cream
V48	Flat to finely folded, rough, opaque, adherent, slightly moist, dark cream
V49	As for A32
V50	Flat, rough, opaque, adherent, slightly moist, grey
V51	Finely folded, rough, opaque, adherent, cream to light pink
V52	Folded, smooth, opaque, moist, cream
V53	Finely folded, rough, opaque, moist, dark cream
V54	As for V29

* strains grown on potato-dextrose agar slants at 30°C for 48 hr.

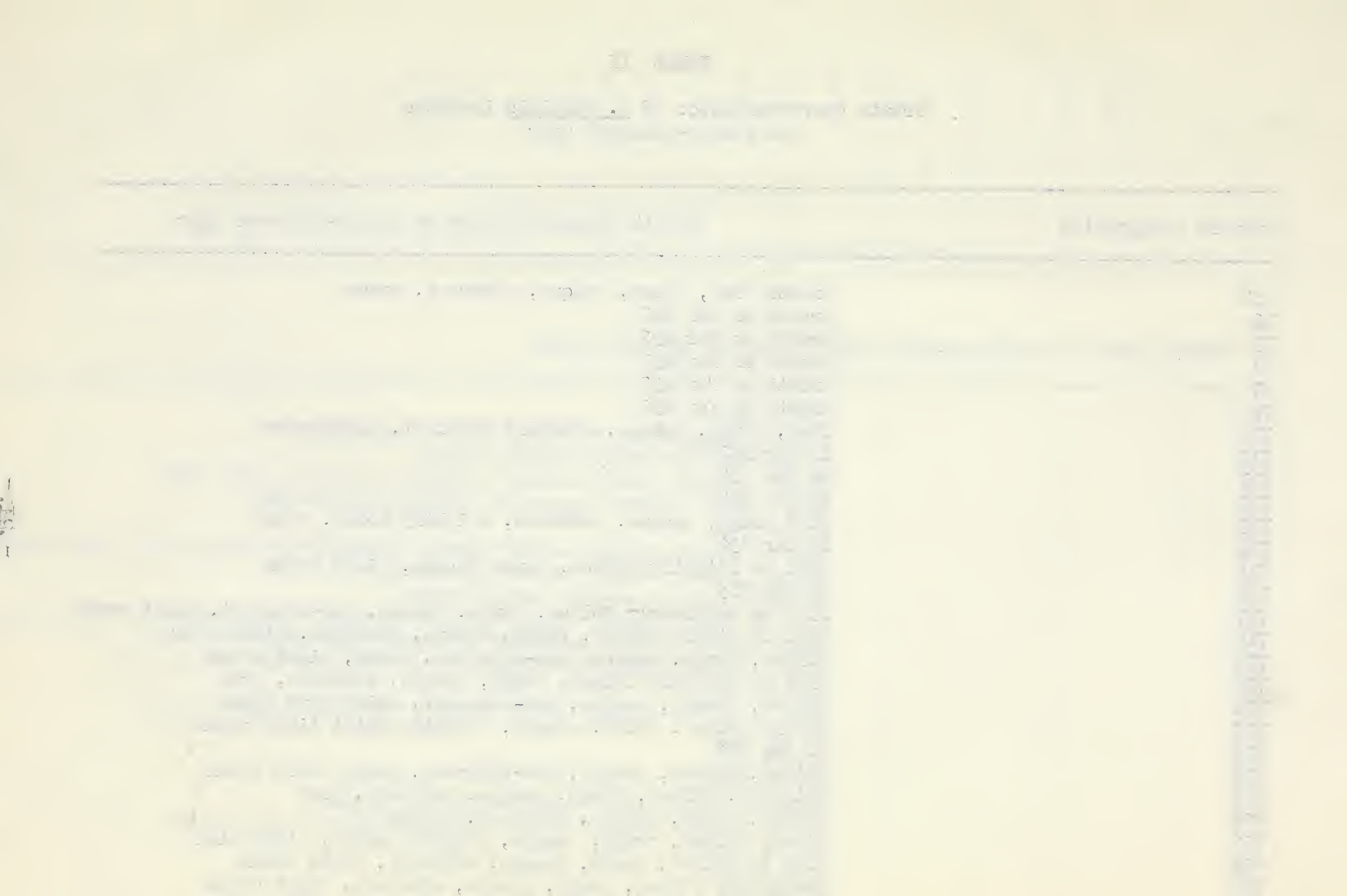

TABLE III

Growth characteristics of B. subtilis isolates on potato slices*

Culture designation	Growth characteristics on potato slices
A32	Growth wrinkled, dry, abundant, cream
V2	Growth as for A32
V3	As for A32
V4	As for A32
V7	As for A32
V8	As for A32
V9	Growth flat to slightly wrinkled, dry, abundant, cream
V10	As for A32
V11	As for A32
V12	As for A32
V13	As for A32
V14	As for A32
V15	Growth finely wrinkled, dry, abundant, pink
V16	Growth warty, dry, abundant, buff
V17	Growth coarsely folded, moist, heavy, off-white
V18	Growth finely wrinkled, dry, abundant, off-white
V19	Growth finely wrinkled, dry, abundant, off-white to black
V20	Growth flat to slightly wrinkled, dry, moderate, off-white
V21	Growth finely wrinkled, dry, abundant, off-white to buff
V22	Growth warty, dry, abundant, cream
V23	Growth coarsely folded, moist, heavy, off-white
V24	Growth flat to slightly warty, moist, abundant, pink
V25	Growth folded, moist, abundant, buff
V26	Growth wrinkled, dry, abundant, yellow
V27	Growth finely wrinkled, dry, abundant, off-white to dark pink
V28	Growth wrinkled, dry, abundant, buff
V29	Growth wrinkled, dry, abundant, pink to off-white
V30	Growth wrinkled, dry, abundant, deep orange
V31	Growth finely wrinkled, dry, abundant, white to off-white
V32	Growth as for V26
V33	Growth as for V26
V34	Growth as for V26
V35	Growth as for A32
V36	Growth flat to slightly wrinkled, dry, abundant, buff
V37	Growth as for A32
V38	Growth as for A32
V39	Growth flat to slightly wrinkled, slightly moist, abundant, light brown
V40	Growth as for V30
V41	Growth as for A32
V42	Growth as for V27
V43	Growth as for A32
V44	Growth flat, dry, abundant, buff
V45	Growth flat, to slightly wrinkled dry, abundant, buff
V46	Growth wrinkled, dry, abundant, pink to dark red
V47	Growth as for V27
V48	Growth very slightly wrinkled, dry, abundant, buff
V49	Growth as for A32
V50	Growth flat, dry, abundant, buff
V51	Growth as for V27
V52	Growth as for V27
V53	Growth as for V27
V54	Growth as for V27

* Strains grown on potato slices at 30°C. for 48 hr.

COMPARATIVE STUDIES ON THE PRODUCTION OF ANTIBIOTICS BY STRAINS OF *B. SUBTILIS*

SPOT-INOCULATION TESTS

Introduction

The ability of an organism to produce an antibiotic may be tested by a number of methods. One which is commonly used is the streak method. Robbins and co-workers (25) used this procedure to test 300 species in 42 genera of the Basidiomycetes for their antibacterial activities. Agar plates which had been seeded with a bacterium were inoculated by streaking inoculum of the fungus across the agar surface. Resultant inhibition of growth of the test bacterium was taken as evidence of the antibacterial property of the fungus under test. Waksman *et al.* (26) tested the antibacterial properties of a large number of actinomycetes by this method.

Antagonism of *B. subtilis* Isolates to *Xanthomonas translucens*

Method

One procedure followed in the present studies in testing the antibiotic properties of *B. subtilis* isolates

was essentially similar to that used in the streak method.

Test plates were prepared by seeding nutrient-dextrose agar* with 1 ml. of a suspension of a bacterial test organism. The suspensions were kept relatively constant by adding a standard loopful of bacteria from a culture of the test organism to 10 ml. of sterile distilled water. One-half ml. of the resulting suspension was added to each plate. To the one-half ml. suspensions were added approximately 15 ml. of melted cooled agar. After the agar was added to the plates they were swirled around to distribute the test organism uniformly throughout the agar and then set aside to harden. When the agar had hardened the plates were spot-inoculated. A loopful of bacteria from a culture of each of the isolates was transferred to the centre of individual test plates. The plates were then incubated at 30°C. Examination of the plates for antibacterial activity was made when growth of the test organism was clearly visible in the plates.

In the first test employing the spot-inoculation method, three strains of a bacterial plant pathogen, Xanthomonas translucens, were used as test organisms. These were Xanthomonas translucens f. sp. cerealis Hagborg (1015), Xanthomonas translucens f. sp. undulosa (S. J. and R.) Hagborg (1018), and Xanthomonas translucens f. sp. cerealis (Hagborg). Each of the 48 isolates tested was spot-inoculated on individual test plates seeded with one of the three test organisms. The A32 strain was included as a standard.

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*Nutrient-dextrose agar: Bacto beef-extract 3 gm., bacto proteose-peptone 5 gm., dextrose 20 gm., distilled water 1000 ml.

The plates were examined for antibacterial activity after 48 hours incubation at 30°C. No attempt was made to accurately measure the degree of inhibition exhibited by the different strains but instead the antibiotic property of each was rated. The results of the test are presented in Table IV.

TABLE IV

Antibiotic activity exhibited by *B. subtilis* isolates against three strains of *Xanthomonas translucens*

Test Organism	Total no. of *B. subtilis* isolates tested	No. of isolates exhibiting different degrees of antibiotic activity — Rating of antibacterial activity*					
		0	1	2	3	4	5
X. translucens (1015)	49	14	1	6	6	17/	5
X. translucens (1018)	49	16	1	4	5	15/	8
X. translucens (cerealis)	49	15	1	5	5	8	15/

* Rating: 0 - no inhibition,
1 - stimulation,
2 - slight inhibition,
3 - moderate inhibition,
4 - marked inhibition,
5 - almost complete to complete inhibition.

/ Indicates the figures given include the A32 strain.

Discussion

It will be seen from the table that considerable variability in antibacterial activity by the B. subtilis isolates is evidenced against the Xanthomonas strains. The variability demonstrated was fairly uniform against each of the test organisms. This is hardly an unexpected result since the test bacteria are strains of a single bacterial species. This property suggests that the physiological processes of each test organism are very similar. Consequently the antibacterial activity exhibited by many of the isolates against one of the pathogens is probably effected through a similar mode of action against the others. This hypothesis is supported by the fact that each of the isolates showed almost the same degree of activity against each of the pathogens.

Those strains whose growth type was smooth in appearance were generally either not active or only weakly antagonistic under the conditions of the test. Four of these, however, produced from the edges of the bacterial colonies, fan-like outgrowths which differed from the central portions of the colonies in presenting a rough type of growth. Wherever these rough sectors occurred in the plates moderate to marked antagonism of the bacterial test organisms was also apparent. It is believed that the change in growth type may result either from an accumulation of metabolic by-products in the medium or possibly from a decrease in the amount of surface moisture present in the plate.

The character of roughness in growth type was

Introduction

associated in each case with those isolates giving the greatest activity against the Xanthomonas strains. However, not all of the rough appearing isolates are active to the same extent.

Antagonism of B. subtilis Isolates to Other Test Bacteria

Robbins and co-workers have reported (25) that of the Basidiomycetes which they studied the slow-growing ones appeared to be the most active antagonists. This question of a possible relation between the rapidity of growth and production of antibiotics was given attention in the present investigations.

Method

The spot-inoculation method was used in studies designed to determine if the antibacterial activity is related to growth rate in B. subtilis. Plates seeded with the test organism were prepared as described above. The test bacteria employed in this test have been designated X - 190, X- 191 and X - 192*. The B. subtilis isolates were tested against each of the test organisms as follows. A standard loopful of bacteria from a culture of an isolate was spotted in four places on the agar surface of each test plate. All spots received approximately the same amount of inoculum. Following their inoculation the plates were in-

* Appendix "A".

associated in each case with those isolates living the greatest activity against the Xanthomonas species. However, not all of the most appearing isolates were active to the same extent.

Antagonism of *P. multifida* Isolates to Other Test Bacteria

Bacillus and co-workers have reported (64) that of the Basidiomycetes which they studied the disc-growing ones appeared to be the most active antagonists. This question of a possible relation between the rapidity of growth and production of antibiotics was given attention in the present investigations.

Method

The spot-inoculation method was used in studies designed to determine if the antibacterial activity is related to growth rate in *P. multifida*. Plates seeded with the test organisms were prepared as described above. The test basidia employed in this test have been designated X - 1007, I- 181 and X - 198*. The *P. multifida* isolates were tested against each of the test organisms as follows. A standard loopful of bacteria from a culture of an isolate was spotted in four places on the edge surface of each test plate. All spots received approximately the same amount of inoculum. Following their inoculation the plates were in-

cubated at 30°C until growth of the test organisms was visible.

It was apparent, on examination of the plates, that a number of the isolates tested were antagonistic to one or more of the test bacteria. This antibacterial activity was evidenced as halos, or clear areas free of growth of the test organism, surrounding the colonies of the active isolates. The size of the inhibition zone in relation to the size of the B. subtilis colony producing it, was taken as a measure of the antibacterial activity exhibited by an isolate against a particular test organism. The results are summarized in Table V.

TABLE V.

Variability in antibacterial activity exhibited by strains of B. subtilis

Test Organism	Medium***	Total No. of Isolates Tested	No. of isolates exhibiting various degrees of inhibition: Degrees of inhibition (mm.)* 0	1-4.9	5-9.9	10-14.9	15-19.9	20
X-190	N.A.	50	17	6	2	16	8+	1
X-191	P.D.A.	48	24	10	1	10+	3	
X-192	P.D.A.	49	22	11	3	12+	1	

* Results based on an average of 4 replicates, measurements made to the nearest 0.5 mm. represent diameter of inhibition zone minus diameter of colony.

*** N. A. - nutrient agar: bacto beef-extract 3 gm., bacto protose-peptone 5 gm., agar 15 gm., distilled water 1000 ml.
P. D. A.- potato-dextrose agar

\+ Indicates figures given includes the A32 strain.

The results show that 54 percent of the isolates tested produced degrees of inhibition greater than 5 mm. against X-190 whereas only 29 and 31 percent of the isolates showed a corresponding amount of inhibition against X-191 and X-192 respectively. These findings cannot be attributed entirely to X-190 being a more sensitive test organism than either X-191 or X-192. The possibility that nutrient agar is a more favorable medium for the production of antibiotic materials by the isolates must also be considered.

The degree of inhibition exhibited by the A32 strain was fairly uniform against each of the test organisms. Three of the isolates however, differed from A32 in the nature of their antibacterial spectra.

TABLE VI.

Antibacterial spectra of B. subtilis isolates

Culture Designation	No. of Replicates	Average degree of inhibition (in mm.)* X-190	X-191	X-192
A32 (1)	4	15.0	10.25	10.25
A32 (2)	4	13.38	12.5	11.38
V2	4	14.38	14.13	15.0
V4	4	13.5	12.63	12.13
V9	4	14.78	2.63	4.38
V33	4	10.25		4.75
V34	4	12.5	3.38	
V35	4	15.88	13.5	14.0

* Values are for four (4) readings.

Position	No. of Replicates	X-191	X-192
A52 (1)	4	16.5	10.26
A52 (2)	4	13.50	18.9
V2	4	14.00	14.75
V4	4	12.5	15.25
V5	4	14.75	9.63
V32	4	10.00	
V34	4	18.5	3.
V36	4	16.00	10.5

* Values are for four (4) replicates.

In Table VI it is quite apparent that the antibacterial spectra of the isolates V9, V33 and V34 differ sharply from that exhibited by the A32 strain. These results suggest that the types of antibiotics produced by these isolates may differ slightly from the active material produced by A32. However, when the media employed in the test are considered, a different explanation is suggested. The low level of activity exhibited by the isolates when they were tested on the potato-dextrose agar medium indicates that there may be present in this medium some factor which limits the amount of active materials produced by the isolates.

The character of the inhibition was found to vary with the isolates. The most pronounced type of inhibition was that in which the B. subtilis colonies were surrounded by a sharply-defined clear zone, indicating complete inhibition of the test bacteria. Other isolates produced zones of inhibition which were limited by zones of stimulation. Both these types have been described (25) by Robbins et al. In some instances, zones of stimulation were apparent which extended beyond that limiting the clear area surrounding the B. subtilis colonies. In these plates the stimulation zones appeared as concentric rings.

The property of antibiotic production was not found to be very closely associated with those isolates which grew rapidly. Generally the more active isolates were also the slower growing forms. Quite often an isolate which had made more growth in one plate than another demonstrated greater

antibacterial activity in the plates containing the lesser amount of growth.

Under the conditions of the tests, considerable variability was shown by the isolates in their ability to produce antibiotically active material. Of those strains capable of exhibiting antibacterial activity, only a few were slightly more active than the A32 strain. However, this relationship varied with the medium and the test organism used. The fact that almost one-half of the isolates produced antibiotically active material is an indication of the active role Bacillus subtilis may play in natural antagonisms.

STUDIES ON THE ANTIBACTERIAL ACTIVITY OF BROTH FILTRATES OF B. SUBTILIS ISOLATES

Introduction

While the spot-inoculation method is a useful procedure for demonstrating the antagonistic properties of a number of isolates, it has certain limitations. The most important of these was experienced in attempts to compare the antibiotic activities of two or more isolates having different growth rates. To overcome this difficulty, the isolates were grown in a liquid medium and the resulting broth cultures were passed through bacterial filters. The bacteria-free filtrates were then tested for antibiotic

activity against several test bacteria.

The antibacterial spectrum of an antibiotic is a record of the degree of activity which it exhibits against a number of test organisms. In addition, such a spectrum is often sufficient to identify the antibiotic.

The following studies, employing culture filtrates of the isolates were undertaken for two purposes. These were to determine if the isolates differed in their ability to produce:

(1) different quantities of a particular antibiotic and (2) different antibiotic compounds.

The filtrates were consequently tested with these purposes in mind by the paper-disc agar-diffusion method of assay.

Paper-Disc Agar-Diffusion Method of Assay

Vincent and Vincent (27) were the first to suggest the paper-disc agar-plate method. It has been widely used in testing culture filtrates of bacteria, actinomycetes, and fungi for antibiotic activity. The method as used in studies on polymyxin has been described by Stansly and Schlosser (28). On analyzing their results statistically, they obtained an error of $\pm$ 15 to 20 percent.

The following procedure, hereafter referred to as the paper-disc agar-diffusion method, was employed to test the activity of culture filtrates of B. subtilis isolates.

Highly absorbent paper discs (Schliecher and Schuell No. 740-E) measuring 12.7 mm. in diameter were sterilized in the autoclave and allowed to dry before being used. Test plates were prepared, as previously described. When the agar had sufficiently hardened the discs were placed on the agar surface. A pair of forceps was used to dip the discs into the filtrates to be tested. Excess solution was allowed to drain from a disc by touching it lightly to the inner surface of the flask containing the filtrate. The disc was then transferred to a test plate. Four discs were similarly prepared for each filtrate, but only two of these were transferred to a plate. Duplicate plates, therefore, of each test organism were prepared for each filtrate. The forceps were flamed between the application of each disc and the cover of the test plate was raised just high enough to allow the disc to be inserted. The plates were then incubated at 30°C until growth of the test organism was clearly visible. Zones of inhibition as evidenced by halos surrounding the discs were taken as indications of a filtrate's antibiotic activity. The widths of the halos were measured to the nearest .5 mm.

Filtration Through Berkefeld Filters

One-hundred ml. lots of .03 percent asparagine medium (18) were dispensed into 500 ml. Erlenmeyer flasks and sterilized for 15 minutes at 15 - 17 lb. pressure.

One ml. of a suspension of an isolate was added to each of three flasks. The suspensions were prepared by adding a standard loopful of bacteria from a culture of an isolate to 10 ml. of sterile distilled water. After the bacteria were added, the flasks were incubated at 30°C for 4 days. The broth cultures of each isolate were then bulked and passed through a Berkefeld filter into sterile receiving flasks. Two sizes of filters (No. 1 and 3) were used. The filtrates of 9 isolates and that of the A32 strain were tested by the paper-disc agar-diffusion method for activity against each of the bacteria shown in Table VII. The filtrates were tested against certain of the test organisms on both nutrient agar and potato-dextrose agar.

Results

As shown in Table VII, the levels of activity exhibited by V3, V8, V10 and V11 are considerably higher than those shown by A32, V2, V4, V7 and V9 for the same test organisms. It will also be noted that the antibacterial spectra of the former differ quite markedly from the spectra of the latter. Since the isolates do not correspond in the pattern of their antibiotic activity towards the various test organisms, the results suggest that the antibiotics produced by V3, V8, V10 and V11 are probably different antibiotic compounds.

The spectrum of the V2 isolate is very similar to the spectrum of A32 when the antibacterial activities

Results

As shown in Table III, the levels of activity exhibited by V3, V6, V10 and V11 are considerably higher than those shown by ESS, V8, V4, V7 and V9 for the test organisms. It will also be noted that the antibacterial spectra of the former differ quite markedly from the spectra of the latter. Since the isolates do not correspond in the pattern of their antibiotic activity somewhat the various test organisms, the results suggest that the antibiotics produced by V3, V6, V10 and V11 are probably different antibiotic compounds.

The spectrum of the V3 isolate is very similar to the spectrum of ESS when the antimicrobial activities

TABLE VII

Antibacterial activity (mm)* of B. subtilis broth culture filtrates**

Test Organism	Culture filtrates of B. subtilis isolates																	
	A32		V2		V3		V4		V7		V8		V9		V10		V12	
	A+	B++	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
X-190	5.63		7.63		11.00		1.38		5.75		10.00		0.38		8.50		8.25	
X-192	1.38	6.75	5.38	7.25	9.38	10.25	0.50	4.00	3.25	7.00	8.88	8.63	0.00	0.75	6.75	8.13	6.63	8.75
B. polymyxa (7070)	1.63	6.13	2.75	6.13	7.63	13.00	0.13	0.70	1.00	4.75	6.38	13.13	0.00	0.00	5.13	10.75	4.75	10.63
X-193	2.13	4.25	2.63	5.13	6.00	9.13	0.00	0.88	1.88	4.50	6.25	8.25	0.00	0.00	4.63	6.88	4.00	6.70
X-191	1.88		5.88		12.50		0.00		1.25		11.50		0.00		10.50		7.75	
X. translucens cerealis+++	1.50		6.00		16.63		0.00		1.50		9.63		0.00		11.00		5.50	
X. translucens (1015)+++	1.50		2.63		9.75		0.00		1.50		9.00		0.00		8.50		5.25	

* Average in mm. of 4 measurements
** Broth cultures filtered through Berkefeld bacterial filters
\+ A - nutrient agar medium
\+\+ B - potato-dextrose agar medium
\+\+\+ Nutrient dextrose agar medium employed

exhibited by these strains are compared. Two marked differences, however, are evidenced when the activities shown towards X-192 and X-191 grown on nutrient agar medium are examined. In these instances, the antibiotic activity exhibited by the V2 culture filtrate is 5 times that shown by A32. These differences suggest that the active material produced by V2 is also somewhat different in type from that produced by the A32 strain.

The degree of activity shown by each of the filtrates was influenced greatly by the medium employed. This was apparent when the filtrates were tested against three test organisms on both nutrient agar and potato-dextrose agar media. In many instances, the degree of activity exhibited by the filtrates on nutrient agar was much less than on potato-dextrose agar.

When the activities of the filtrates are considered on the basis of the amount of antibiotic present marked variability is apparent, Figure 1 and Figure 2. Two of the filtrates contained very little antibiotic whereas the others apparently contained varying amounts. It is not possible, however, to attribute these results to differences in the ability of the isolates to produce different amounts of a particular antibiotic. In subsequent studies, employing the Berkefeld filters, it was discovered that the filters adsorbed, or inactivated, appreciable amounts of active material. This was made apparent when filtrates, which had become contaminated, were refiltered to remove the contaminant. Filtrates possessing certain levels of activity when tested following a second

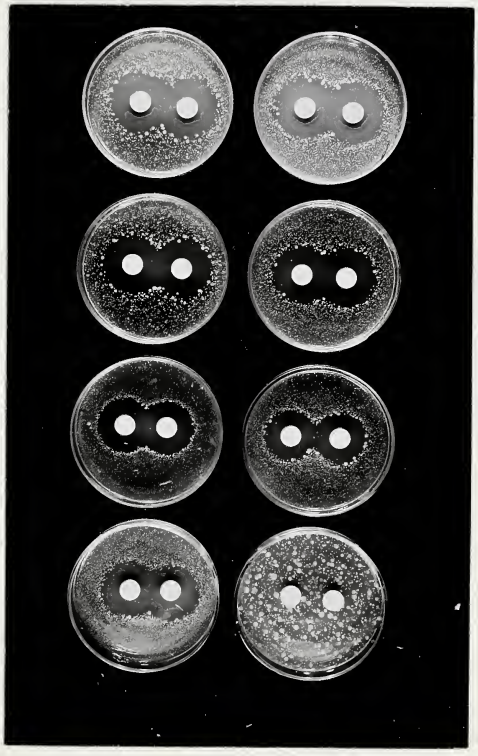


Figure 1

Antibacterial activity exhibited by broth culture filtrates of *B. subtilis* isolates towards X-190

Row on left, top to bottom: V12, V8, V3, A32
Row on right, top to bottom: V10, V7, V2, Ck.

Figure 2

Antibacterial activity exhibited by broth culture filtrates of B. subtilis isolates towards Xanthomonas translucens cerealis

Row on left, top to bottom: V12, V8, V3, A32
Row on right, top to bottom: V10, V7, V2, Ck.

filtration were found to be completely inactive. As two different sizes of filters were employed in the test, varying amounts of active material may have been removed.

Goodman (18) has shown that Nuchar, an activated charcoal, alumina and soil effectively adsorb the antibiotic material produced by the A32 strain. Other substances tested reduced the antibiotic activity of broth cultures to varying degrees. The diatomaceous earth, of which Berkefeld filters are composed, was found in the present studies to be another material which readily adsorbs A32 antibiotic.

Filtration Through Seitz Filters

The Seitz bacterial filter, the filtering part of which consists essentially of a filter-paper pad, was also used to measure the degree of activity produced by B. subtilis isolates in a broth medium. Flasks containing .03 percent asparagine medium were inoculated with suspensions of the isolates and incubated at 30°C for 4 days. Three flasks, each containing 100 ml. of medium were prepared for each isolate. Following the incubation period, the cultures of each isolate were bulked and passed through a Seitz filter. The filtrates were then tested for activity against various test organisms. The values shown in Table VIII represent the average inhibition exhibited by B. subtilis isolates towards each of the test bacteria. Four measurements,

TABLE VIII

Antibacterial activity (mm.)* of B. subtilis broth culture filtrates**

Test Organism	Broth filtrates of B. subtilis									
	A32	V2	V3	V4	V7	V8	V9	V10	V12	L.S.D.
X-190	10.75	9.75	11.38	11.38	11.0	12.0	0.0	12.5	11.75	.75
X. translucens (1015)	5.38	2.13	5.63	4.25	5.5	7.5	0.0	7.63	6.0	1.04
X. translucens (1017)	5.38	1.5	6.63	5.0	6.38	7.63	0.0	10.13	7.88	1.27
X. translucens (1907)	10.5	6.88	10.75	9.38	10.63	11.63	0.0	13.88	12.75	.82
X. translucens (1018)	7.63	3.88	7.13	6.25	8.25	8.25	0.0	11.5	9.0	1.12
X. translucens cerealis	7.0	4.38	7.38	5.63	7.38	7.88	0.0	10.5	8.25	.83
X. translucens (R5)	6.5	1.5	6.38	4.5	5.75	8.13	0.0	9.88	6.88	.65
B. polymyxa 7070	7.13	5.88	7.63	7.25	7.5	8.88	0.0	9.13	9.88	.17
X-191	9.5	8.63	10.13	9.5	9.0	10.25	.625	11.125	10.5	.72
X-193	8.25	6.63	8.75	7.75	8.38	9.13	0.0	10.25	9.13	.18

* Values represent averages each based on 4 discs

** 300 ml. of culture liquid filtered through Seitz filters

TABLE VIII

Multicollinearity Matrix (Part 2 of 2). Symbols Above Column Figures Refer to Table V

Zero Tolerance of 1.0 Indicates

Head Segment	Age	Sex	V2	V3	V4	V7	V8	V9	Vpa	Vpb	Vpc	Vpd
1. Temporalis (TEM)	1.000	.212	.078	.000	.000	.000	.000	.000	.000	.000	.000	.000
2. Masseter (MAS)	.212	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
3. Pterygoideus medialis (PTM)	.078	.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
4. Digastricus (DIG)	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000
5. Pterygoideus lateralis (PTL)	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000	.000
6. Temporalis anterior (TEA)	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000
7. Temporalis posterior (TEP)	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000
8. Temporalis middle (TEM)	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000
9. Masseter superficialis (MAS)	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000
10. Masseter profundus (MAP)	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000
11. Pterygoideus medialis (PTM)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	.000
12. Pterygoideus lateralis (PTL)	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000

Head Segment Key: 1-Temporalis (TEM), 2-Masseter (MAS), 3-Pterygoideus medialis (PTM), 4-Digastricus (DIG), 5-Pterygoideus lateralis (PTL), 6-Temporalis anterior (TEA), 7-Temporalis posterior (TEP), 8-Temporalis middle (TEM), 9-Masseter superficialis (MAS), 10-Masseter profundus (MAP), 11-Pterygoideus medialis (PTM), 12-Pterygoideus lateralis (PTL)

Based on 1 degree of freedom between variables (Wilks, 1932)

TABLE IX

A comparison of antibacterial activity of broth filtrates of B. subtilis isolates and A32

Test Organism	Ratio of activity*								
	A32	V2	V3	V4	V7	V8	V9	V10	V12
X-190	1.0	.91	1.06	1.06	1.02	1.12	-- **	1.16	1.15
X. translucens (1015)	1.0	.40	1.05	.79	1.02	1.40	--	1.42	1.16
X. translucens (1017)	1.0	.28	1.23	.93	1.19	1.42	--	1.88	1.47
X. translucens (1907)	1.0	.15	1.02	.89	1.01	1.11	--	1.32	1.21
X. translucens (1018)	1.0	.51	1.07	.82	1.08	1.08	--	1.51	1.18
X. translucens cerealis	1.0	.63	1.05	.80	1.05	1.13	--	1.50	1.18
X. translucens (R5)	1.0	.27	.98	.69	.89	1.25	--	1.52	1.06
B. polymyxa 7070	1.0	.83	1.07	1.02	1.05	1.25	--	1.28	1.39
X-191	1.0	.91	1.07	1.0	.95	1.08	--	1.17	1.11
X-193	1.0	.80	1.06	.94	1.02	1.11	--	1.24	1.06

* Ratio = $\frac{\text{mm. inhibition exhibited by isolate}}{\text{mm. inhibition exhibited by A32 strain}}$

** Dash indicates no activity exhibited

TABLE IX

A comparison of sanitoconial sorptivity of bronz silicates
at 25° subphotilla isotope method 452

(data to other)

Test Organism	SCA	CA	Cy	Mg	Ag	Mn	Ca	Mn
Coli-Aero	1.0	0.7	0.0	1.00	0.0	0.0	0.0	0.0
M. tuberculosis (str)	1.0	0.0	0.0	0.0

one for each of four discs, were obtained by measuring the widths of the halos surrounding the discs. In Table IX the results are expressed as ratios which are obtained by comparing the average inhibition shown by each isolate towards a particular test organism to that exhibited by the A32 strain against the same test organism.

Further studies employing broth filtrates differred from the foregoing test not only in the isolates which were tested but also in the quantities of culture liquid which were filtered. Smaller quantities of medium were employed in order to shorten the time required for filtration. Results of these studies are presented in Table X and in Table XI. In one test (Table X), 200 ml. of broth culture of the isolates were filtered and tested for antibiotic activity. In another (Table XI), only 100 ml. of culture liquid were filtered.

Discussion

The variability in antibiotic production exhibited by the isolates, as shown in Table VIII and Table IX, may result not only from the amount of antibiotic produced but also from the type or types of antibiotic produced. Some of the isolates appear to produce the same type of antibiotic as that which is produced by the A32 strain. This is apparent when the antibacterial spectra of V3, V4 and V7 are examined, since the degree of activity shown by the isolates is almost the same as that shown by A32, towards the same test organisms.

TABLE X

Antibacterial activity (mm.)* of
B. subtilis broth culture filtrates**

Test Organism	Broth filtrates of B. subtilis			
	A32	V11	V13	V14
X-190	11.13	10.13	13.63	12.13
Xanthomonas translucens (1015)	1.38	1.75	4.25	2.13
Xanthomonas translucens (1017)	1.0	1.5	5.25	2.0
Xanthomonas translucens (1907)	4.63	5.25	6.75	4.0
Xanthomonas translucens (1018)	2.5	2.5	6.13	3.5
Xanthomonas translucens (R5)	3.0	3.0	6.75	4.38
Xanthomonas translucens cerealis	1.25	2.5	5.88	4.25
B. polymyxa 7070	7.25	7.75	9.38	8.13
X-191	7.0	6.88	7.63	7.25
X-192	8.5	9.0	10.5	10.0

* Values represent averages, each based on 4 discs

** 200 ml. of culture liquid filtered through a Seitz filter.

TABLE XI

Antibacterial activity (mm.)* of *B. subtilis* broth culture filtrates**

Test Organism	Broth filtrates of *B. subtilis*				
	A32	V16	V20	V25	V28
X-190	8.5	7.5	6.38	4.88	4.63
Xanthomonas translucens (1015)	0.0	1.75	2.125	0.75	0.0
Xanthomonas translucens (1017)	0.0	1.0	trace	0.0	0.0
Xanthomonas transluces (1907)	0.0	4.5	5.25	3.5	trace
Xanthomonas translucens (1018)	0.0	2.63	3.5	1.38	0.0
Xanthomonas translucens (R5)	0.0	3.0	2.88	1.0	0.75
Xanthomonas translucens cerealis	0.0	3.5	4.5	1.38	0.0
B. polymyxa 7070	1.13	2.38	4.12		
X-191	2.38	5.0	5.63	4.75	3.0
X-192	0.0	4.75	5.88	4.75	2.13

* Values represent averages each based on 4 discs

** 100 ml. of culture liquid filtered through Seitz filter.

On this basis, a significatn difference in the degree of activity shown by V3, V4 and V7 probably represents, under the conditions of the test, a difference only in the amount of a particular antibiotic produced.

The isolates V2 and V10, however, exhibit degrees of activity which are markedly different from that of A32 against the various test bacteria. In these instances the differences which are significant may be best explained on the basis that V2 and V10 produce antibiotics which are different in type to the active material produced by A32.

The fact that the filtrate of V9 was completely inactive towards almost all of the test organisms suggests that this isolate does not produce antibiotics active against the test organisms used. However, results presented in a subsequent section of the text indicate that the Seitz filter adsorbs all of the active material produced by this isolate. This would occur if either the quantity of antibiotic produced were limited or if the antibiotic type compound were more readily adsorbed by the filter than those of other isolates.

The antibiotic activity shown by V8 and V12 toward certain of the test organisms is significantly different from that of the A32 strain. The differences, however, are not so marked as those which are apparent on comparing V2 and V10 with A32. Although the pattern shown by V8 and V12 suggests that the differences are related to a difference in the type of antibiotic produced, a comparison of these results with those of other tests is necessary to support this hypothesis.

It will be noted, in Table X, that the degree of antibacterial activity exhibited by V13 contrasts markedly with that shown by A32 against certain of the test bacteria. In three instances, V13 was almost 5 times more active than A32 against the same test organisms. These large differences in antibiotic activity against some of the test organisms and the almost equal degrees of activity shown toward others, suggests that there is a chemical difference in the type of antibiotic produced by these strains. V11 and V14, however, under the conditions of the test, appear to be very similar to A32 in the nature of their antibiotic activity.

From the results presented in Table XI, additional evidence of variability in antibiotic production by the isolates is shown. The isolates V16, V20 and V25 apparently possess antibacterial spectra which differ from that of the A32 strain. These differences in the antibacterial spectra suggest that the isolates V16, V20 and V25 also produce antibiotics somewhat different in type to the active material produced by A32. The antibiotic activity exhibited by V28, however, is similar to that shown by A32 towards the test bacteria; the active material produced by these strains is therefore probably of the same type.

When the three tables of results, Table VIII, Table X and Table XI, are compared on the basis of the degrees of activity exhibited by the A32 strain in each test, a marked reduction in antibiotic activity is apparent. This reduction is associated with the smaller quantities of culture liquid which were employed. From these results and the

results presented in a subsequent section of the text, it is quite evident that appreciable amounts of antibiotic material are removed by the Seitz filter when culture liquids are passed through it. Relatively larger amounts of active material are removed when small quantities of culture liquid are filtered than when larger volumes are filtered.

STUDIES ON THE ANTIBACTERIAL ACTIVITY OF BROTH CULTURES OF *B. SUBTILIS* ISOLATES

Penicylinder-cup Method of Assay

Broth cultures which had not undergone any treatment likely to modify antibiotic material present in them were tested by the penicylinder-cup method of assay.

Test plates were prepared by adding 15-ml. of melted, cooled agar to Petri plates containing a one-half ml. suspension of a test organism. The plates were swirled to distribute the test organism uniformly throughout the agar medium. When the agar had sufficiently hardened sterile penicylinder-cups were transferred with a pair of forceps to each plate. The so-called cups are small cylinders, measuring 8.5 mm. in diameter, which are open at both ends. The tapered end of a cup was placed on the agar surface and by gentle pressure it was pressed into the agar. Two plates,

each plate containing two cups, were used for each isolate tested.

The isolates were grown in 0.03 percent asparagine medium for 4 days at a temperature of 30°C. Samples of the culture liquids were pipetted into the cups from 1-ml. pipettes. Only enough medium was added to the cups to just fill them. The plates were then kept at 5°C for approximately 6 hours after which time they were incubated at 30°C until growth of the test organism was clearly visible.

The period in which the plates were kept at 5°C provided an interval in which growth of the test organism was at a minimum. This afforded a longer period for diffusion of the active material through the agar and resulted in larger zones of inhibition.

In a preliminary trial 50 isolates were tested for antibiotic activity against three test organisms, X-190, X-191 and B. polymyxa. The degrees of inhibition shown by the isolates towards X-190 are given in Table XII. Those isolates which were either inactive or only weakly active against X-190 showed a similar result when tested against X-191 and B. polymyxa. Several of the isolates, however, when tested against X-191 and B. polymyxa, exhibited appreciable degrees of activity. These isolates were re-examined for their antibiotic properties using the penicylinder-cup method. Results of this test are given in Table XIII.

TABLE XII

Antibacterial activity (mm.)* of broth cultures of B. subtilis isolates

Culture Designation	Inhibition (mm.) of X-190	Culture Designation	Inhibition (mm.) of X-190	Culture Designation	Inhibition (mm.) of X-190
A32	33.42	V20	24.0	V38	24.5
V2	37.58	V21	8.5	V39	23.0
V3	32.58	V22	8.5	V40	27.5
V4	40.58	V23	8.5	V41	29.25
V7	35.08	V24	8.5	V42	18.25***
V8	33.0	V25	33.0	V43	8.5
V9	35.83	V26	33.8	V44	8.5
V10	40.5	V27	8.5	V45	8.5
V11	32.5	V28	40.5	V46	11.5
V12	31.0	V29	13.0	V47	21.42***
V13	34.0	V30	31.5	V48	29.0
V14	29.0	V31	8.5	V49	36.0
V15	8.5**	V32	18.33***	V50	16.0
V16	31.0	V34	31.0	V51	13.0
V17	31.25	V35	32.33	V52	8.5
V18	8.5	V36	12.25	V53	8.5
V19	15.0	V37	17.0 ***	V54	8.5
				Broth Ck.	8.5

* Values are averages for 4 cups
Diameter of inhibition zone measured in mm.

** Value represents diameter of cup in mm.

*** Indicates that the inhibition zone was not clearly defined

TABLE 12

Antibacterial activity (in % of
growth without) of _E. faecalis_ treated

TABLE XIII

Antibacterial activity (mm.)* of
broth cultures of B. subtilis isolates

Culture Designation	Degrees of activity of broth cultures against: X-190	X-191	B. polymyxa 7070
A32	31.50	38.25	37.38
V2	29.63	35.25	32.25
V3	30.88	33.38	29.63
V4	30.38	31.0	33.50
V7	32.0	35.75	32.50
V8	32.88	31.13	32.25
V9	24.0	23.75	16.75
V10	28.63	34.75	26.0
V11	29.0	33.63	31.50
V12	27.50	31.25	27.88
V14	27.0	31.75	27.0
V16	29.0	---	27.13
V20	27.50	28.0	31.0
V25	25.75	29.33	26.75
V26	8.50**	14.25	8.50
V28	28.75	35.88	32.0
V30	18.50	28.67	19.63
V31	8.50	9.0	8.50
V34	26.50	27.50	22.75
V35	31.38	37.75	32.13
V38	8.50	10.63	8.50
V39	27.0	28.38	26.63
V40	25.5	29.0	30.75
V41	26.38	28.75	27.5
V48	25.38	31.25	22.75
V49	30.75	33.63	34.50
Broth Check	8.50	8.50	8.50

* Values are averages for 4 cups
Diameter of inhibition zone measured in mm.

** Value represents diameter of cup measured in mm.

TABLE XIII

Antibacterial activity (mm.)* of broth cultures of B. subtilis isolates

Designation	Culture	X-180	X-181	B. subtilis
Check		8.50	8.50	8.50
V48		30.75	33.25	34.75
V49		25.25	31.25	28.75
V40		24.00	26.75	27.0
V41		25.5	25.0	30.75
V38		8.50	10.00	8.50
V39		27.0	20.50	20.75
V34		25.50	27.50	32.75
V35		31.25	37.75	28.12
V36		19.50	26.67	14.66
V31		8.50	8.0	8.50
V24		25.50	35.75	26.0
V28		26.75	29.25	25.75
V26		8.50**	14.25	8.50
V25		25.75	29.25	25.75
V20		27.50	24.0	21.0
V16		23.0	---	27.75
V14		27.0	21.75	27.0
V12		27.50	21.25	27.50
V11		29.0	25.25	21.50
V10		24.50	21.25	24.25
V9		24.0	25.75	19.75
V8		20.25	24.75	20.0
V7		24.0	20.75	23.25
V6		30.25	21.0	26.50
V5		30.38	21.0	26.50
V4		30.88	25.25	24.25
V3		24.25	26.25	26.25
V2		27.50	26.25	24.25
V1		27.50	26.25	27.50

* Values are averages for 4 cups
Diameter of inhibition zone measured in mm.

** Value represents diameter of cup measured in mm.

Discussion

The results shown in Table XII compare very closely with the results obtained when the isolates were tested for antibiotic activity by the spot-inoculation method (Table V). The broth culture studies show that, of the 50 isolates tested, 26 of them or 52 percent produce appreciable amounts of antibiotic material as evidenced by degrees of inhibition of X-190 measuring more than 20 mm. When the isolates were tested against this organism in the spot-inoculation tests, 54 percent of those tested showed what are considered to be comparable degrees of activity.

Under the conditions of the broth culture test 14 isolates showed no antibacterial activity towards X-190. Six isolates were weakly active, producing inhibition zones of 16 mm. or less. In 4 instances, the inhibition zones were quite large. However, some growth of the test organism was apparent within the zones. This type of inhibition is described as diffuse since the test organism's growth was not completely inhibited within the inhibition zone.

When the more active isolates are compared on the basis of their antibacterial activity against three test organisms (Table XIII), variability in antibiotic production is again evidenced. The degrees of activity shown by the isolates towards X-190 agree quite closely with the activity shown by them in the preliminary test (Table XII). The generally lower values observed for most of the isolates are

Discussion

probably a result of the shorter period in which the plates were kept at 5°C. In the preliminary test the plates were held for 9 hours at 5°C, whereas, on the second test the plates were at the lower temperature for only 6 hours. Two of the isolates, V26 and V38, were much less active when tested the second time. As shown in Table XIII, they are only very weakly active against the test bacteria employed. Subsequent tests showed a similar result which indicates that the antibiotic properties of these organisms have been modified. This indicates that the antibiotic properties of these isolates are not very stable. Artificial culture of the isolates may have caused the loss of the property for antibiotic production.

The test plates of X-191 and B. polymyxa were kept for 9 hours at 5°C. By comparing the antibacterial activities of the isolates against these two test organisms, differences are observed which indicate that the types of antibiotic material produced by a few of the isolates probably differs from the active material produced by A32. Four of the isolates V9, V10, V30 and V48 show a marked reduction in the degrees of activity shown towards B. polymyxa from that shown towards X-191. This behavior is decidedly different from that of A32 which is highly antagonistic to both of these test organisms. This implies that there is a chemical difference between the types of antibiotic formed by each of the four isolates and that produced by the A32 strain.

COMPARATIVE STUDIES ON THE ANTIBACTERIAL ACTIVITIES OF BROTH CULTURES AND BROTH FILTRATES

Comparison of Berkefeld Culture Filtrates with Seitz Culture Filtrates

The following procedure was employed to determine the relative amounts of adsorption of antibiotic material from broth cultures of A32 by Berkefeld and Seitz bacterial filters. One-hundred ml. of asparagine broth medium were added to 500 ml. Erlenmeyer flasks. The flasks were then sterilized in the autoclave for 15 minutes. Two kinds of inocula, pasteurized and non-pasteurized were used in the test. These were included to determine the effect of such inocula on antibiotic production by A32. Suspensions of inoculum were prepared by transferring loopfuls of bacteria from a culture of A32 to test-tubes of sterile 0.85 percent saline solution. One loopful of bacteria was added to each of three test-tubes containing 10 ml. of saline solution. One of the tubes was then placed in a water-bath where it was kept for 10 minutes at a temperature of 80°C. This treatment, commonly known as pasteurization, has the effect of killing the vegetative cells of bacteria. It does not, however, inactivate the spores of those bacteria capable of forming them. Following the heat treatment of one of the suspensions, one ml. of the pasteurized suspension and a like quantity of the non-pasteurized suspension was added

to individual flasks containing asparagine medium. A total of three flasks for each treatment were prepared. A single flask containing broth medium, to which 1-ml. of saline solution had been added, served as a check. All of the flasks were incubated at 30°C for 4 days. Following the incubation period, the contents of two flasks of each treatment were bulked. One-half of the resulting culture liquid was passed through a Berkefeld filter. The remainder was filtered through a Seitz filter. Culture filtrates were then tested by the paper-disc agar-diffusion method of assay. The results are given in Table XIV. Neither of the check preparations exhibited antibacterial activity against the test organism used in the test.

TABLE XIV

Comparison of the antibacterial activities of Berkefeld and Seitz culture filtrates of B. subtilis A32

Type of Filter	Kind of Inoculum	Antibacterial activity (mm.)* against X-190
Berkefeld	Pasteurized	6.5
	Non-pasteurized	6.5
Seitz	Pasteurized	13.0
	Non-pasteurized	12.5

* Values are averages of 4 readings.

Discussion

It is clearly evident that the Berkefeld filter removes considerable amounts of antibiotic material from broth cultures of A32. It is further evident that this property of the filter assumes added importance when organisms are compared on the basis of the amount of antibiotic they produce. Those organisms which produce antibiotic material that is readily adsorbed by the filter would be considered less active than others whose antibiotic is not adsorbed to the same extent.

The results also show that there was no effect on the amount of antibiotic produced by cultures of A32 when two kinds of inocula namely pasteurized and non-pasteurized were employed.

Removal of Antibiotic Material from *B. subtilis* Broth Cultures by Seitz Filters

In a preliminary test, broth cultures of *B. subtilis* isolates were filtered through Seitz bacterial filters. The culture filtrates and the non-filtered broth cultures were then tested for antibiotic activity by the paper-disc agar-diffusion method. Cultures of the isolates were prepared by adding one-ml. suspensions of each of the isolates to individual flasks of asparagine broth. Two flasks were prepared for each isolate and all of the flasks were incub-

ated at 30°C for 4 days. Following the incubation period, the broth cultures of each isolate were bulked and one-half of the resulting culture liquid was filtered through a Seitz filter. Both the filtered and non-filtered culture liquids of each isolate were then tested for antibiotic activity. The test organism employed in the test was X-190. Two plates, each plate containing 2 discs, were used to test each culture liquid. In those test plates containing discs which had been dipped in the non-filtered lots of broth, growth of the isolate was apparent from some of the discs. Other discs were sufficiently free of growth, however, to permit readings of the amount of adsorption which was evidenced. The results are presented in Table XV.

TABLE XV

Comparison of the antibacterial activities of Seitz culture filtrates and broth cultures of B. subtilis isolates

Isolate Tested	Growth (mm.)** from disc	Activity (mm.)* of culture liquids towards X-190	
		Broth Culture	Broth Filtrate
A32	trace	11.0	3.5
V3	trace	12.0	2.5
V8	1.0	12.0	5.5
V10	trace	12.5	6.5
V14	1.0	10.0	2.0
V16	2.0	11.0	1.0
V17	2.0	12.0	4.5
V35	1.0	12.0	4.5
V38	6.0	8.0	0.0
V39	5.0	7.5	0.0
V40	3.5	10.5	0.0
V41	4.0	13.5	2.5
V49	0.0	11.0	6.0

* Values are averages for 4 discs
** Values are averages of 4 measurements. Discs used were the same as those in column 2.

232	[illegible]	11.0	[illegible]
93	[illegible]	1.0	[illegible]
[illegible]	1.0	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	1.0	[illegible]	[illegible]
[illegible]	2.0	[illegible]	[illegible]
[illegible]	2.0	12.0	[illegible]
[illegible]	1.0	[illegible]	[illegible]
[illegible]	0.0	[illegible]	[illegible]
[illegible]	0.0	[illegible]	[illegible]
[illegible]	1.0	[illegible]	[illegible]
[illegible]	1.0	[illegible]	[illegible]
[illegible]	0.0	[illegible]	[illegible]

Discussion

It will be seen from the table that the Seitz filter removes appreciable amounts of antibiotic material from broth cultures of B. subtilis isolates. In those instances where growth from the disc was very extensive, it is not possible to relate the reduction in activity to adsorption by the filter. A part of the activity evidenced when the broth cultures were tested may have resulted from antibiotic that was produced during the period in which the plates were incubated. However, if the results for the isolates A32, V3, V10 and V49 are examined, it is quite evident that the filter was responsible for a loss of antibiotic material from culture liquors of these isolates.

Adsorption of A32 Antibiotic by Seitz Filters

In the studies employing broth culture filtrates, it was noted that Seitz filters appeared to remove relatively greater amounts of antibiotic material from small volumes of broth cultures than from larger volumes. The following procedure was used to determine the amount of antibiotic material removed by the filter when successive 50-ml. lots of broth were filtered. Flasks of asparagine medium were inoculated with 1-ml. suspensions of B. subtilis

A32. They were then incubated for 4 days at 30°C. Following the incubation period the contents of three of the flasks were bulked to give 300-ml. of culture liquid. This volume of liquid was then passed through a Seitz filter in 50-ml. lots with each lot of filtrate caught in a separate receiving flask. The container of the filter was allowed to empty completely before an additional lot of culture liquid was added to it. When all of the lots of broth had been filtered they were tested for antibiotic activity by the penicylinder-cup method of assay. Culture liquid which had not been filtered was also tested.

TABLE XVI

Relative amounts of antibiotic adsorbed by a Seitz filter from successive 50-ml. lots of broth culture

Volume (mm.) of culture liquid filtered	Antibacterial activity (mm.)* shown against X-190
0	38.12
50	21.88
100	31.5
150	31.75
200	30.75
250	31.13
300	28.58

* Values are averages for 4 cups.

Relative amounts of acidity in filtrates of a
Seitz filter (pre-sterilized [illegible] type of
sterile culture

Volume (cc.) of filtrate collected	[illegible] [illegible] (cc.)*
0	[illegible]
50	[illegible]
100	[illegible]
150	[illegible]
200	[illegible]
250	[illegible]
300	[illegible]

* [illegible] were standard [illegible].

Discussion

It will be seen from the table that a relatively greater amount of antibiotic material is removed from the first 50-ml. of culture liquid than from successive 50-ml. lots. After the first 100-ml. of liquid have passed through the filter, the amount of antibiotic removed becomes almost constant when more liquid is filtered. One possible explanation of these results is that broth cultures of A32 may contain more than one antibiotic. When the first 50-ml. lot of liquor is filtered, the filter removes both types of antibiotic material from the culture liquid. On the filtration of further quantities of liquor, the filter becomes saturated with respect to one component but not the other. Filtration of additional lots of medium, therefore, effects a removal of antibiotic material of only one type. Such an effect might be evidenced if the two components were present in different proportions.

Comparison of the Antibacterial Activities of Broth Cultures and Broth Filtrates

Benedict and Stodola (29) have reported that polymyxin from crude culture liquor is highly adsorbed by Seitz filter pads. Antibiotic material of B. subtilis isolates has also been shown to be adsorbed from broth culture liquors. The variability evidenced on testing

Discussion

the culture filtrates suggests that differences in antibacterial activity may have resulted from differences in the amounts of antibiotic adsorbed. When broth cultures of the isolates were tested, the degrees of antibacterial activity exhibited by many of the isolates were quite uniform. If similar amounts of antibiotic were removed by the filters from broth cultures of the isolates, the degrees of antibacterial activity of the filtrates should show the same uniformity. This would be particularly true if the active materials produced by the isolates were the same. The following procedure was used to test this hypothesis.

One-hundred ml. of asparagine broth medium were dispensed into 500-ml. Erlenmeyer flasks. The flasks were sterilized in the autoclave, allowed to cool, and then inoculated with 1-ml. suspensions of the isolates. Two flasks were prepared for each isolate. Following an incubation period of 4 days at 30°C, the contents of replicate flasks of each isolate were bulked. The bulked lots of culture liquid were then tested for antibacterial activity against X-191. A total of 25 strains, including the A32 strain, were tested by the penicylinder-cup method for antibiotic activity. Following the addition of samples of the culture liquor to the cups, the plates were kept at 5°C for 6 hours. They were then transferred to a 30°C incubator where they remained for 16 hours. The degrees of activity of the different isolates are shown in Table XVII. The figures shown represent averages of 4 readings for each isolate; the meas-

TABLE XVII

Comparative degrees of antibacterial activity evidenced by broth cultures and broth culture filtrates* of B. subtilis isolates against X-191

Culture Designation	Inhibition (mm) evidenced by: Broth Culture	Broth Filtrate	Ratio: Inhibition (mm.) by isolate / Inhibition (mm.) by A32 Broth Culture	Broth Filtrate
A32	25.25	21.88	1.0	1.0
V2	26.88	21.0	1.06	.96
V3	25.38	14.75	1.01	.67
V4	21.5	15.88	.85	.73
V7	22.75	17.63	.90	.81
V8	23.00	18.63	.91	.85
V10	24.13	20.75	.96	.95
V11**	24.00	17.88	.96	.82
V12	24.75	13.13	.98	.60
V13	26.00	19.13	1.03	.87
V14**	24.38	9.75	.97	.45
V16	19.5	10.25	.77	.47
V17	25.25	17.5	1.0	.80
V20	19.25	9.0	.76	.41
V25	19.63	9.38	.78	.43
V26	13.88	8.5	.55	.39
V28	23.25	15.88	.92	.73
V30	19.13	9.88	.76	.45
V35	23.88	22.88	.96	1.06
V38	15.0	9.0	.59	.41
V39	18.13	8.5	.72	.39
V40	23.25	17.38	.92	.79
V41	27.38	21.25	1.08	.97
V48	23.5	14.5	.93	.66
V49	26.38	18.0	1.04	.82
Check	8.5	8.5	.34	.39
LSD	3.25	2.72		

* Obtained after filtration through a Seitz filter.

** One-hundred ml. of culture liquid filtered.

urements were made to the nearest 0.5 mm.

After the culture liquids were tested, as described above, they were passed through Seitz bacterial filters. As only two filters having equal capacities were available, the culture liquors of the isolates were kept at 2°C until such time as they could be filtered. Following individual filtrations, the filter-pads, whose identities were maintained, were removed to sterile Petri plates. The filters were thoroughly washed, rinsed in distilled water and sterilized prior to each filtration. Following the filtration of a culture liquor, the filtrate was transferred to the low temperature. When all of the culture liquids had been filtered, they were tested by the procedure used in testing the broth cultures. The periods at which the plates were kept at 5°C and 30°C were also 6 and 16 hours respectively. The degrees of activity exhibited by broth filtrates of the isolates are also given in Table XVII. A simple analysis of variance was made for each set of results. The least significant differences (L.S.D.) obtained are included in the table.

Discussion

The loss in activity exhibited by A32 on filtering culture liquor through a Seitz filter (Table XVII) is comparable to that which was lost in a previous test

measured were made to the nearest 0.5 mm.

After the culture liquids were tested, as described above, they were passed through sets of sintered glass filters. As only two filters having equal porosities were available, the culture filtrate of the isolates were kept at 30° until such time as they could be filtered. Following individual filtrations, the filter-cells, whose sterilities were maintained, were removed to sterile Petri plates. The filters were thoroughly washed, rinsed in distilled water and sterilized prior to each filtration. Following the filtration of a culture liquor, the filtrate was transferred to the ice temperature. When all of the culture liquids had been filtered, they were tested by the procedure used in testing the broth cultures. The periods at which the plates were kept at 6°C and 30°C were also 6 and 24 hours respectively. The degrees of activity exhibited by broth filtrates of the isolates are also given in Table XVII. A simple analysis of variance was made for each set of results. The latest significant differences (L.S.D.) obtained are included in the table.

Discussion

The zone in activity exhibited by ACS in sterile culture liquor through a Berka filter (Table XVII) is comparable to that which was lost in a previous test

(Table XVI) when the same volume of liquid was filtered. As shown in Table XVII, the amount of active material removed from the A32 culture liquid as compared to the amounts removed from broth cultures of many of the isolates is relatively small (Figure 3). Marked variability in the degrees of antibacterial activity evidenced on testing the culture filtrates is apparent. The relative degrees of activity exhibited by the broth cultures, however, are much more uniform.

Eight isolates differ from the A32 strain by a significant amount when the degrees of activity exhibited by the broth cultures are compared. Twenty of the 24 isolates tested show a significant difference when the degrees of activity exhibited by broth filtrates are compared. The lack of uniformity in the relative degrees of activity shown by the filtrates suggests that the active materials produced by many of the isolates are antibiotic compounds which differ in type from that produced by A32.

Standard errors inherent in the penicylinder-cup method of assay varying from $\pm$ 4 percent to $\pm$ 30 percent, depending on the number of replicates used and the degree to chich the techniques employed were standardized, have been reported (30). Pure antibiotic preparations of known concentrations were included whenever broth cultures were tested for antibiotic activity. As pure A32 antibiotic was not available for comparison, in the studies reported here, the method of assay employed is not strictly analagous to those which have been reported. For this reason, only those

(Table XVI) when the same volume of liquid was filtered.
As shown in Table XVII, the amount of active material re-
moved from the ASO culture fluid as compared to the am-
ounts removed from broth cultures of many of the isolates
is relatively small (Figure 8). Another variability in
the degree of antibacterial activity evidenced on heating
the culture filtrates is apparent. The relative degrees
of activity exhibited by the broth cultures, however, are
much more uniform.

Eight isolates differ from the ASO strain by a
significant amount when the degrees of activity exhibited
by the broth cultures are compared. Twenty of the 24
isolates tested show a significant difference when the
degrees of activity exhibited by broth filtrates are com-
pared. The lack of uniformity in the relative degrees of
activity shown by the filtrates suggests that the active
materials produced by many of the isolates are antibiotic
compounds which differ in type from that produced by ASO.

Standard errors incurred in the penicillinase-cup
method of assay varying from 2 4 percent to $\pm$ 20 percent,
depending on the number of replicates used and the degree to
which the techniques employed were standardized, have
been reported (20). Three antibiotic preparations of known
concentrations were incorporated whenever broth culture was
tested for antibiotic activity. As pure ASO antibiotic was
not available for comparison, in the studies reported here,
the method of assay employed is not strictly analogous
to those which have been reported. Use will, only

Figure 3

Relative activity of non-filtered and Seitz filtered broth cultures of B. subtilis A32 and B. subtilis V3 towards X-191

Top row, left to right: non-filtered broth cultures of V3 and A32

Bottom row, left to right: Seitz filtered broth culture of V3 and A32

isolates which showed marked differences from the A32 strain are considered on the basis of the type of antibiotic produced. B. subtilis strains which evidenced such a difference are V3 (Figure 3), V12, V14, V16, V20, V25, V30 and V48.

Because only 100-ml. of broth culture of the V14 isolate were filtered, results shown for this isolate are not comparable to those given for the A32 strain. The quantity of V11 culture filtrate which was tested, however, was the same as the quantity tested of V14. Since the degree of antibacterial activity of the broth filtrate of V14 is much less than that of V11, these isolates also appear to differ in the types of antibiotic compound which they produce.

Antibacterial Activity Evidenced on Testing Seitz Filter-Pads for Adsorbed Antibiotic

The individual filter-pads through which the culture liquids of the B. subtilis isolates had been filtered were tested for antibiotic material. Small bits of the pad, removed from the under-side, were placed on the surface of agar test plates. Two such pieces of an air-dried pad were transferred to test plates of X-190. The plates were incubated overnight and measurements for antibiotic activity were made the next day. The diameter of inhibition zones which were apparent around many of the

papers was measured in mm. The width of the paper was subtracted from the diameter of the inhibition zones. The results of the test are given in Table XVIII.

TABLE XVIII

Antibacterial activity evidenced by active material adsorbed by Seitz filter-pads

Culture designation	Inhibition (mm.) of X-190*	Width (mm.) of paper tested**	Difference in mm.
A32	26.0	5.5	20.5
V2	33.0	6.5	26.5
V3	36.0	8.0	28.0
V4	35.5	6.0	29.5
V7	30.5	6.5	24.0
V8	31.0	5.5	25.5
V10	31.5	5.0	26.5
V11	32.5	6.5	26.0
V12	21.0	7.0	14.0
V13	30.0	5.5	24.5
V14/	13.0	5.0	8.0
V16	20.0	7.5	13.5
V17	26.0	7.0	19.0
V20	0.0		0.0
V25	21.0	6.0	15.0
V28	25.0	7.0	18.0
V30	trace		trace
V35	30.5	6.5	24.0
V38	0.0		0.0
V39	trace		trace
V40	23.5	6.0	17.5
V41	31.5	7.0	24.5
V48	22.5	5.5	17.0
V49	27.0	5.5	21.5

* Averages of two measurements

** Averages of two measurements

/ Only one reading made

Culture designation	Inhibition (mm.) of X-120*	Width (mm.) paper tested**	Difference in mm.
V32	25.0		
V2	33.0		
V3	35.0		
V4	35.0		
V7	35.0		
V6	31.0		
V10	31.0		
V11	35.0		
V12	21.0		
V14	30.0		
V15	15.0		
V16	29.0		
V17	25.0		
V20	8.0		
V22	21.0	6.0	15.0
V29	25.0	7.0	18.0
V30	Clear		Clear
V33	30.5	6.5	24.0
V28	0.0		0.0
V39	Clear		Clear
V40	23.5	6.0	17.5
V41	31.0	7.0	24.0
V48	25.5	8.0	17.5
V49	27.0	8.0	19.0

* Only one reading made

** Average of two measurements

† Average of two measurements

Antibacterial activity evidenced by native material extracted by water filtrates

TABLE XVIII

Results of the test are given in Table XVIII.

subtracted from the diameter of the inhibited zones. The papers were measured in mm. The width of the paper was

Discussion

Although the areas of the bits of paper which were transferred to the plates were not constant, the inhibition zones developed within individual test plates were quite uniform in size. The largest difference apparent between diameters of two zones within a single plate was 8 mm. the average difference exhibited for any one plate was 2 mm.

It will be apparent from the results given in Table XVIII that the antibiotic produced by many of the isolates is not permanently adsorbed by the filter pad. In a few instances, though active material was removed from the culture liquid by the filter, no inhibition of the test organism was evidenced on testing the filter-pad. This suggests that the active material is either permanently adsorbed or inactivated by the filter.

On comparing the results shown for V11 and V14, it is evident that the antibiotic material produced by V11 is considerably more active than that of the V14 isolate. In the foregoing test, an apparently greater amount of active material was removed from the broth culture of V14 than from the culture liquor of V11. The amount of active material released by the filter-pad was considerably less than would be expected on the basis of the relative amounts adsorbed. This is additional evidence in support of the hypothesis that the active materials of V11 and V14 are

different antibiotic compounds.

The degree of activity evidenced by V12 active material, on testing the filter-pad, is much less than that exhibited for A32. Although more antibiotic was removed from the culture liquid of V12, considerably less is shown in the filter-pad test plates. These results suggest that there is a difference in the antibiotic type compounds produced by these strains.

EXTRACTION STUDIES OF THE ANTIBIOTIC MATERIAL PRODUCED BY B. SUBTILIS ISOLATES

Extraction Procedure

One hundred ml. of .03 percent asparagine medium were dispensed into 500-ml. Erlenmeyer flasks. The flasks were then sterilized in an autoclave for 15 minutes. Suspensions of B. subtilis isolates, using a loopful of bacteria of each isolate in 10 ml. of sterile distilled water, were prepared. One ml. of a suspension was added to each of 15 flasks. The flasks were then incubated at 30°C for 4 days. Following the incubation period, the broth cultures were bulked giving a total of 1500 ml. of broth culture for each isolate.

The culture liquids were then extracted 3 times with n-butanol. Enough butanol was added for each extraction

to form a layer after the mixtures were shaken. A volume of butanol equal to 15 percent of the total volume of culture liquid extracted was used for the three extractions. Following the third extraction the mixtures were saturated with sodium chloride and were then warmed slightly. This had the effect of causing most of the residual butanol to separate out. The method differs slightly from that employed by Goodman (18). One modification consisted in not removing the bacterial growth from the culture liquid. This was not done for two reasons. Firstly, previous studies had shown that both the Berkefeld and the Seitz bacterial filters removed varying amounts of antibiotic from the cultures. Secondly, the subjection of the bacterial cells to extraction with a solvent might remove antibiotic material which was held within the cells. Another difference consisted in not extracting the culture liquid with petroleum-ether prior to the butanol extraction.

The broth layers following the butanol extraction were tested for antibiotic activity by the paper-disc agar-diffusion method. Only traces of activity were found to be present.

The butanol extracts were treated, in part, according to the method described by Gordon (19) for purification of the antibiotic of B. subtilis A32. The butanol extract, together with an added equal volume of water, was distilled in vacuo until all the butanol was removed. The distillation temperature was about 25°C. The resulting water suspension was shaken three times with chloroform. Five-ml. of chloro-

to form a layer after the mixtures were shaken. A volume of butanol equal to 15 percent of the total volume of culture liquid extracted was used for the three extractions. Following the third extraction the mixtures were saturated with sodium chloride and were then warmed slightly. This had the effect of causing most of the residual butanol to separate out. The method differs slightly from that employed by Goodman (18). One modification consisted in not removing the bacterial growth from the culture liquid. This was not done for two reasons. Firstly, previous studies had shown that both the Berkefeld and the Seitz bacterial filters removed varying amounts of antibiotic from the culture. Secondly, the adsorption of the bacterial cells to extraction with a solvent might remove antibiotic material which was held within the cells. Another difference consisted in not extracting the culture liquid with carbon tetrachloride prior to the butanol extraction.

The broth layers following the butanol extraction were tested for antibiotic activity by the paper-disc agar-diffusion method. Only traces of activity were found to be present.

The butanol extracts were treated, in part, according to the method described by Goodman (18) for purification of the antibiotic of B. subtilis ASP. The butanol extract, together with an added equal volume of water, was distilled in vacuo until all the butanol was removed. The distillation temperature was about 25°C. The resulting water suspension was shaken three times with chloroform. Five-ml. of chloro-

form were used for each extraction. The mixture formed on the addition of the chloroform, when left overnight, separated into layers. The water layer from the third chloroform extraction was centrifuged at approximately 4500 r.p.m. for 30 minutes. This effectively reduced the number of bacterial cells held in suspension and resulted in more accurate readings when the water layer was tested for antibiotic activity, as it was immediately following centrifugation.

Only those isolates that produced appreciable amounts of antibiotic, as determined by the penicylinder-cup test, were tested by the above procedure. None of these, however, showed any activity when the water layers following a chloroform extraction were tested.

Discussion

The procedure described above is apparently a satisfactory one for the extraction and partial purification of antibiotic material produced by the B. subtilis isolates studied. Since only traces of activity were apparent when the water layers were tested, it would appear that the active material of each isolate has certain similar properties.

Tests Employing Chloroform Extracts

Chloroform extracts of the isolates were tested by the paper-disc agar-diffusion method against several test or-

Discussion

The procedures described above is apparently a satisfactory one for the extraction and initial purification of antibiotic material produced by the *B. subtilis* isolates studied. Since only traces of activity were found in any case when the water layer was tested, it would appear that the active material of each isolate has certain similar properties.

Tests Employing Ultraviolet Irradiation

Ultraviolet extracts of the isolates were tested by the paper-disc diffusion method against several test or-

ganisms. The results are summarized in Table XIX and Table XX. The results represent separate tests and they are, therefore, presented in separate tables.

TABLE XIX

Antibacterial activity of chloroform extracts of *B. subtilis* isolates

Test organism	Activity (mm)* exhibited by chloroform extracts.				
	A32	V13	V10	V16	V35
X-191	11.5	12.0	9.5	9.5	9.0
X-193	12.0	11.5	4.0	8.0	3.0
X-194	9.0	8.0	4.5	3.0	3.0
B. polymyxa 7070	12.0	9.0	3.0	6.5	3.0
X-195	0.0	1.0	0.0	2.5	0.0
X-196	0.0	0.0	0.0	2.0	0.0
X-197	0.0	0.0	0.0	0.0	0.0
X-198	2.5	3.5	10.0** (diffuse)	3.5	9.0 (diffuse)

* Figures are averages of 4 readings

** Inhibition zones were not completely free of growth of the test organism.

Antibacterial activity of chloroform extracts of *B. subtilis* isolates

Test organism	ASS	YLS	CLO	CLE	VSS
X-121	11.5	18.0	8.5	9.5	7.0
X-122	12.0	11.5	4.0	8.0	6.0
X-124	9.0	0.0	8.5	0.0	0.0
B. polymyxa 7010	12.0	0.0	5.0	0.0	0.0
X-126	0.0	5.0	0.0	8.5	0.0
X-130	0.0	0.0	0.0	0.0	0.0
X-127	0.0	0.0	0.0	0.0	0.0
X-128	8.5	5.5	10.0^{**}	5.5	0.0

Activity (mm)* exhibited by chloroform extracts.

(Filter paper)

* Figures are averages of 4 readings

** Inhibition zones were not completely free of growth of the test organism.

TABLE XX

Antibacterial activity of chloroform extracts of B. subtilis isolates

Test Organism	Activity (mm.)* exhibited by chloroform extracts					
	A32	V14	V17	V39	V41	V49
X-191	11.0	10.5	10.5	9.5	10.5	11.0
X-193	9.0	9.0	6.5	9.0	9.5	8.0
X-195	1.5	3.5	5.0	5.0	3.0	3.5
B. polymyxa 7070	7.5	7.5	5.0	8.0	8.0	7.0
X-196	3.0	4.0	2.0	2.0	1.0	4.0
X-197	0.0	0.0	trace	trace	0.0	0.0
X-198	4.5	6.0	3.0	6.0	3.0	4.5

* Figures are averages of 4 readings

Discussion

The activities of the chloroform extracts show considerable variation in the degree of inhibition they exhibit against the test bacteria (Figure 4). It will be noted from an examination of Table XIX that the antibacterial spectra of V10, V16 and V35 are different from the spectra of A32 and V3. This is most evident when the degrees of inhibition towards X-193, B. polymyxa and X-194 are compared with the activity shown towards X-191. The inhibition evidenced against X-191 by all of the extracts is uniformly high.

TABLE IX

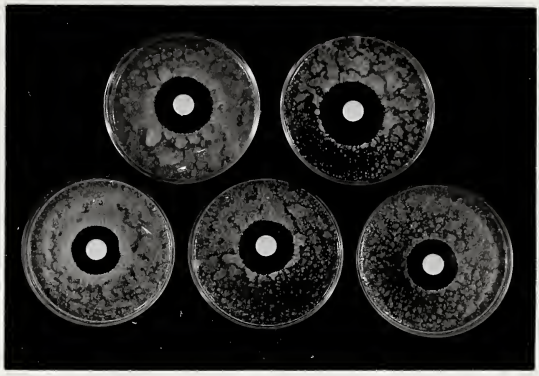
Antibacterial activity of chloroform extracts of *B. subtilis* isolates

Test Organism	Activity (mm.)* exhibited by chloroform extracts					
	A32	V16	V17	V3	V42	V49
X-192	11.0	10.5	10.5	9.5	10.5	11.0
X-193	9.0	9.0	8.5	9.0	9.5	9.0
X-195	1.5	3.5	5.0	4.5	3.0	3.5
B. polymyxa 7070	7.5	7.5	5.0	6.0	6.0	7.0
X-196	3.0	4.0	2.0	2.5	4.0	4.0
X-197	0.0	0.0	Trace	Trace	0.0	0.0
X-199	4.5	6.0	3.0	5.0	3.0	4.5

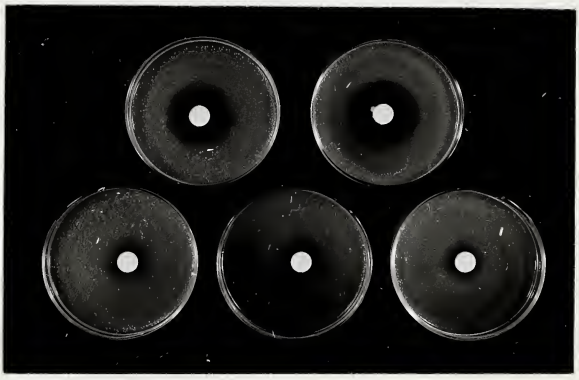
* Figures are averages of 4 readings

Discussion

The activities of the chloroform extracts show considerable variation in the degree of inhibition they exhibit against the test bacteria (Figure 4). It will be noted from an examination of Table IX that the antibacterial spectra of V16, V18 and V35 are different from the spectra of A32 and V3. This is most evident when the degree of inhibition towards X-193, *B. polymyxa* and X-195 are compared with the activity shown towards X-192. The inhibition evi-



A



B

Figure 4

Antibacterial activity of chloroform extracts of B. subtilis isolates

A Towards X-191
Top row, left to right: A32, V3
Bottom row, left to right: V10, V16, V35

B Towards X-193
Top row, left to right: A32, V3
Bottom row, left to right: V10, V16, V35

However, the degree of activity shown towards the other test organisms varies from marked inhibition by the A32 and V3 extracts, to only slight inhibition by V10 and V35, and moderate inhibition by V16. These results are further evidence that different isolates may produce different antibiotic type compounds.

A consideration of the results presented in Table XX shows that the differences in the antibacterial spectra of the isolates tested are not very great. On the basis of this test therefore, it would appear that the antibiotics produced by the isolates V14, V17, V39, V41 and V49 are very similar to that produced by the A32 strain.

Considerable difficulty was experienced when further tests employing chloroform extracts other than those recorded in Tables XIX and XX were performed. Not only the chloroform extracts but also chloroform check preparations showed considerable variation. This variation was evidenced in repeated trials and also between replicate discs within a particular test. It was assumed that the variation was possibly due to the use of different batches of chloroform. The presence of a toxic factor in one of them could account for the inconsistency of the results. However, definite proof in support of this assumption was not obtained.

Tests Employing Ethanol Solution

Chloroform extracts of B. subtilis isolates were evaporated to dryness under vacuum. The resulting solids were brown in color and in some of them crystalline structure was apparent. The addition of 30 ml. of 95 percent ethanol brought only a part of the solid material into solution. White-colored residues remained which were separated from the amber-colored solutions by filtration through Whatman #1 filter paper. Following this treatment the ethanol solutions were transferred to a 5°C refrigerator in which they were stored until all of the chloroform extracts had undergone the procedure described above. The periods the solutions were held at the low temperature prior to testing them for activity varied from 2 days to 3 weeks. The penicylinder-cup method of assay was employed in testing the solutions for antibiotic activity. The white residues and the filter papers used in filtering the solutions were tested by transferring small amounts of each to agar plates seeded with a test organism. X-190 was employed as the test bacterium. Three plates, each containing two cups, were used in testing the solution of each isolate. The figures presented in Table XXI are averages of six readings made for each isolate tested. None of the white residues or the filter papers tested showed any activity.

Tests Employing Ethanol Extraction

Chloroform extracts of B. subtilis 6633 were evaporated to dryness under vacuum. The resulting solids were brown in color and in case of tube cultivation were apparent. The addition of 35 ml. of 95 percent ethanol brought only a part of the solid material into solution. White-colored residues remained which were separated from the ether-colored solution by filtration through Whatman #1 filter paper. Following this procedure the ether solutions were transferred to a CO_2 refrigerator in which they were stored until all of the fractions extracts had undergone the procedure described above. The petroleum ether solutions were held at the low temperature prior to testing them for activity varied from 2 days to 3 weeks. The petroleum-ether-soluble portion of assay was employed in testing the solutions for antibiotic activity. The White paper-disc and the filter papers used in filtering the solutions were tested by transferring small amounts of each to open plates seeded with a test organism. X-ray was employed to the test medium. Three plates, each containing two tubes, were used in testing the solution of each isolate. The figures presented in Table XVI are averages of six readings made for each isolate tested. None of the tests resulted in the filter papers tested showed any activity.

TABLE XXI

Antibacterial activity of ethanol solutions of *B. subtilis* isolates tested against X-190

Ethanol extract of isolates	Av. inhibition (mm)* of test bacterium X-190	Ethanol extract of isolates	Av. inhibition (mm) of test bacterium X-190
A32 - 1	28.0	**V26	8.5
A32 - 2	31.37	V29	20.17
V2	23.25	V39	24.35
V14	27.83	**V40	8.5
V17	15.17	V41	21.7
V19	8.5	V49	26.83
		***Ethanol check	8.5

* Values are averages of 6 cups. Diameter of inhibition zone measured in mm.
** Flocculent precipitate present in test solution.
*** Value is the diameter of the cup measured in mm.

Discussion

At the time the extraction studies were initiated, no satisfactory method was available for testing the antibiotic activity of broth cultures prior to chemical extraction. Consequently only by comparing the activities of the isolates as shown in the broth culture studies (Table XII and Table XIII) with the activities exhibited by the ethanol solutions (Table XXI) is it possible to determine the effect of the various chemical treatments on the antibiotic material

produced by the isolates.

The degree of antibiotic activity shown by each of the isolates V2, V14, V17, V39, V41 and V49 is apparently only slightly modified by the extraction procedure. Antibiotic material produced by V17 and V40 was apparently very much affected by the chemical treatments employed. It will be noted, however, that a flocculent precipitate was present in the ethanol solutions of V26 and V40 when the test was performed. Precipitation also occurred in the ethanol solutions of V2, V14, V19 and V39 following an interval of about 2 weeks. During this period and for about 2 weeks prior to the test, the solutions were kept at a temperature of 5°C. Following the transfer of the flasks from the low temperature to room temperature, only one flask, after approximately 24 hours at the higher temperature, had a precipitate remaining in it. When the solutions were subsequently tested for activity against X-190, X191 and B. polymyxa there was considerable variability evidenced in replicate test plates of some of the solutions. Solutions of A32, V2 and V29 exhibited degrees of antibiotic activity which were fairly uniform between replicate plates in a particular test. However, reduction in activity was evidenced when further tests were performed. These results suggest, that under the conditions of the test, the antibiotic materials of the isolates tested were modified in some way in the presence of the ethanol. As the ethanol solutions were not all prepared at one time, the antibiotics of each of the isolates may have been at different stages of inact-

produced by the isolates.

The degree of antibiotic activity show by each of the isolates V2, V14, V17, V29, V41 and V49 is apparently only slightly modified by the extraction procedure. Antibiotic material produced by V17 and V40 was apparently very much affected by the chemical treatments employed. It will be noted, however, that a flocculent precipitate was present in the ethanol solutions of V29 and V40 when the test was performed. Precipitation also occurred in the ethanol solutions of V2, V14, V19 and V29 following an interval of about 2 weeks. During this period and for about 2 weeks prior to the test, the solutions were kept at a temperature of 50C. Following the transfer of the flasks from the low temperature to room temperature, only one flask, after approximately 24 hours at the higher temperature, had a precipitate remaining in it. When the solutions were subsequently tested for activity against S-120, K-4 and B. polymyxa there was considerable variability evidenced in replicate test plates of some of the solutions. Solutions of A32, V2 and V29 exhibited degrees of antibiotic activity which were fairly uniform between replicate plates in a particular test. However, reduction in activity was evidenced when further tests were performed. These results suggest, that under the conditions of the test, the antibiotic materials of the isolates tested were modified in some way in the presence of the ethanol. As the ethanol solutions were not all prepared at one time, the satisfaction of each of the isolates may have been at different stages of inact-

ivation when the tests were performed. It is not possible, therefore, to relate the differences in antibiotic activity shown in Table XXI to differences in properties of the antibiotics entirely.

The degree of activity shown by the V29 isolate is this study is very much different from the activity shown by it in the spot-inoculation and broth culture studies. This result suggests that there is active material contained in the cells of this isolate which is released during the extraction procedure. Jansen and Herschmann (7) have found that subtilin is obtained as an antibiotic product of the interior of the cells of a strain of B. subtilis. The active material is removed from the cells by a chemical extraction procedure.

ASSOCIATION OF CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS WITH THE ANTIBIOTIC PROPERTIES OF B. SUBTILIS

The association of a cultural or physiological characteristic with the ability of an organism to produce antibiotics would greatly facilitate the discovery of new antibiotic-producing strains. With this in mind, the following characters of the B. subtilis isolates were studied in relation to the ability of the isolates to produce antibiotic material.

ivation when the tests were performed. It is not possible, therefore, to relate the differences in morphologic activity shown in Table XXI to differences in properties of the antibiotics entirely.

The degree of activity shown by the VRS isolate in this study is very much different from the activity shown by it in the agar-inoculation and broth culture studies. This result suggests that there is active material contained in the cells of this isolate which is released during the extraction procedure. Jawson and Gerencser (7) have found that subtilin is obtained as an antibiotic product of the interior of the cells of a strain of B. subtilis. The active material is removed from the cells by a chemical extraction procedure.

ASSOCIATION OF CHARACTERS AND DIFFERENTIATION
CHARACTERIZATION WITH THE ANTIBIOTIC
PRODUCTION OF B. SUBTILIS

The association of a cultural or physiological characteristic with the ability of an organism to produce antibiotics would greatly facilitate the discovery of new antibiotic-producing strains. With this in mind, the following characters of the B. subtilis isolates were studied in relation to the ability of the isolates to produce antibiotic material.

Growth Type

Growth of the isolates on potato-dextrose agar slants showed considerable variation in type. When compared in relation to their ability to produce antibiotic material in broth culture, the most active strains were found to have a rough type of growth (Table XXII). Not all of the rough strains, however, were antibiotically active. Isolates whose growth was smooth were either only weakly active or inactive.

Color of the Isolates

The isolates were grown on potato-dextrose agar slants and on potato slices. Following their incubation at 30°C the cultures were examined for the production of pigments. While both of the media supported pigment formation, the potato slices gave slightly more intense colors than the potato-dextrose agar slants. The isolates were compared on the basis of color in relation to antibiotic activity (Table XXII). Cultures exhibiting a pink coloration are either only weakly active or not active in the production of antibiotic material. The most active isolates are those whose cream color is similar to that of the A32 strain. Other strains, however, exhibiting this color are among those isolates which produce no active material when grown in broth culture. Two strains which are orange in color

Growth Type

Growth of the isolates on potato-dextrose agar plates showed considerable variation in type. When compared in relation to their ability to produce fruiting material in broth culture, the most active screens were found to have a rough type of growth (Table XIII). Not all of the rough strains, however, were equally active. Isolates whose growth was smooth were either only weakly active or inactive.

Color of the Isolates

The isolates were grown on potato-dextrose agar plates and on potato slices. Following their incubation at 30°C the cultures were examined for the production of pigments. While both of the media supported pigment formation, the potato slices gave slightly more intense colors than the potato-dextrose agar plates. The isolates were compared on the basis of color in relation to antibiotic activity (Table XIII). Cultures exhibiting a pink coloration were either only weakly active or not active in the production of antibiotic material. The most active isolates are those whose green color is similar to that of the AFS strain. Other strains, however, exhibiting this color are found among those isolates which produce no active material. Two strains which are orange in color in broth culture. Two strains which are orange in color

produce appreciable amounts of antibiotic. Four yellow strains have been very variable throughout the investigations in their ability to produce antibiotically active material. The property of producing active material which they possessed during the earlier studies appears to have been lost. Such behavior has also been reported for strains of Penicillium chrysogenum (31).

Reaction to Alcoholic Ferric Chloride

Jansen and Hirschmann (7) found B. subtilis isolates possessing a high subtilin activity to give an intense blue color, and those with little subtilin activity no color, on the addition of a few drops of a one percent alcoholic solution of ferric chloride to 5 ml. of broth culture. In the studies reported here, none of the isolates gave a blue color with ferric chloride which suggests that none of the strains tested, under the conditions of the test, produces subtilin. Some of the cultures, however, as a result of the treatment showed a layering effect with the material in the top layer having a coagulated appearance. This characteristic was found to be closely associated with weak antibiotic activity.

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TABLE XXII

Physiological and morphological properties of isolates of B.subtilis in relation to antibiotic activity

Culture Designation	Activity against (mm)* X-190	Physiological and morphological properties				
		Fermentation of lactose**	p H of lactose broth/	Alcoholic $FeCl_3$ test//	Growth type	Pigment produced on Potato slices
A32	33.41	pos.	6.52	neg,	rough	cream
V2	31.58	pos.	6.51	neg.	rough	cream
V3	32.58	pos.	6.52	neg.	rough	cream
V4	40.58	pos.	6.51	neg.	rough	cream
V7	35.08	pos.	6.51	neg.	rough	cream
V8	33.0	pos.	6.51	neg.	rough	cream
V9	35.83	pos.	6.62	neg.	rough	cream
V10	40.5	pos.	6.51	neg.	rough	cream
V11	32.5	pos.	6.50	neg.	rough	cream
V12	31.0	pos.	6.52	neg.	rough	cream
V13	34.0	pos.	6.52	neg.	rough	cream
V14	29.0	pos.	6.52	neg.	rough	cream
V15	nil	neg.	6.60	neg.	rough	pink
V16	31.0	pos.	5.99	neg.	rough	buff
V17	31.25	pos.	6.52	neg.	rough	off-white
V18	nil	neg.	6.61	neg.	rough	off-white
V19	15.0	neg.	6.62	neg.	rough	off-white
V20	24.0	pos.	6.52	neg.	rough	off-white
V21	nil	neg.	6.62	neg.	smooth	off-white
V22	nil	neg.	6.62	neg.	rough	cream
V23	nil	neg.	6.69	neg.	smooth	off-white
V24	nil	neg.	6.62	coag.	smooth	pink
V25	33.0	pos.	6.51	neg.	rough	buff
V26	33.83	neg.	6.64	neg.	rough	yellow
V27	nil	neg.	6.62	coag.	rough	pink
V28	40.5	pos.	6.51	neg.	rough	buff
V29	13.0	neg.	----	coag.	rough	pink
V30	31.5	pos.	6.54	neg.	rough	orange
V31	nil ///	neg.	6.63	neg.	rough	white
V32	18.33(diffuse)	neg.	6.61	coag.	rough	yellow
V33		neg.	----	neg.	rough	yellow
V34	31.0	pos.	6.50	neg.	rough	yellow
V35	32.33	pos.	6.51	neg.	rough	cream
V36	12.25	neg.	6.62	coag.	rough	buff
V37	17.0(diffuse)	neg.	6.61	neg.	rough	cream
V38	24.5	pos.	6.52	neg.	rough	cream
V39	23.0	pos.	----	neg.	rough	brown
V40	27.5	pos.	6.51	neg.	rough	orange
V41	29.25	pos.	6.51	neg.	rough	cream
V42	18.25(diffuse)	neg.	6.62	coag.	rough	pink
V43	nil	neg.	6.61	coag.	smooth	cream
V44	nil	neg.	6.61	neg.	rough	buff
V45	nil	neg.	6.61	coag.	rough	buff
V46	11.5	neg.	6.61	neg.	smooth	pink
V47	21.41(diffuse)	neg.	6.65	coag.	rough	pink
V48	29.0	pos.	6.69	neg.	rough	buff
V49	36.0	pos.	6.51	neg.	rough	cream
V50	16.0	neg.	6.60	neg.	rough	buff
V51	13.0	neg.	6.69	neg.	rough	pink
V52	nil	neg.	6.60	coag.	smooth	pink
V53	nil	neg.	6.70	coag.	rough	pink
V54	nil	neg.	6.7	neg.	rough	pink

* Values are averages of 4 cups: Diameter of zone of inhibition measured in mm.
** Positive reading indicates acid production
/ p H determined on 4-day-old lactose broth cultures: Measured with Beckman p H meter.
// few drops of .5% alcoholic chloride solution added to broth culture negative reading indicates no blue color formed; coag. indicates coagulation apparent as a result of the treatment
/// inhibition not clearly defined, some growth by test organism in inhibition zone

Carbohydrate Fermentation

In the identification studies, the isolates were tested for their ability to utilize a number of sugars. The sugars employed in the tests were xylose maltose, galactose, lactose, dextrose and sucrose. Some of the isolates were found to be incapable of utilizing galactose and lactose. However, all of the isolates fermented xylose, maltose, dextrose and sucrose. An ammoniacal-nitrogen medium was used for the fermentation studies. It was made up as follows:

Ammoniacal-nitrogen medium

$NH_4H_2PO_4$	- 1 gm.
KCl	- 0.2 gm.
$MgSO_4$	- 0.2 gm.
Agar	- 13.0 gm.
Distilled water	- 1000 ml.

The pH was adjusted to pH 7.0, and 12 ml. of an 0.04 percent solution of bromcresol purple were added as an indicator. After tubing and autoclaving, a solution of the test carbohydrate, sterilized separately by filtration through a Seitz filter, was pipetted into each tube in such quantity as to result in an 0.5 percent concentration of the carbohydrate. The tubes were incubated overnight and examined for contamination. They were then inoculated and observed for growth, acid production, and gas, after 2 and 14 days. The production of acid by an isolate was

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Ammonium-nitrogen medium

$NH_4H_2PO_4$	- 1 gm.
KCl	- 0.2 gm.
$MgSO_4$	- 0.2 gm.
Agar	- 15.0 gm.
Distilled water	- 1000 ml.

The pH was adjusted to pH 7.0, and 12 ml. of an 0.04 percent solution of bromocresol purple were added as an indicator. After boiling and autoclaving, a solution of the test carbohydrate, sterilized separately by filtration through a Seitz filter, was pipetted into each tube in such quantity as to result in an 0.5 percent concentration of the carbohydrate. The tubes were inoculated overnight and examined for contamination. They were then incubated and observed for growth, acid production, and gas, after 2 and 14 days. The production of acid by an isolate was

taken as evidence of the ability of that isolate to utilize the test sugar. The change in color of the agar from purple to yellow indicated acid production.

The results of the tests show (Table XXII) that a very high degree of association exists between the ability of an isolate to produce appreciable amounts of antibiotic material and its ability to ferment lactose. In almost every instance, those isolates which do not utilize lactose are also not active or only weakly active. However, those isolates which utilize lactose, as evidenced by the production of acid, also produce appreciable amounts of antibiotic. Forty-seven of the 52 cultures tested showed this relationship at the time the tests were performed. The other 5 isolates were later found to also conform to the earlier results.

When the isolates were grown in lactose broth a very high degree of association between the ability of an isolate to produce appreciable amounts of antibiotic material and its ability to lower the pH of the broth was observed. The broth was prepared by essentially the same procedure as that followed in preparing the lactose agar. However, neither agar nor bromcresol purple were included in the broth medium. Tubes of the medium were inoculated with the B. subtilis strains after which they were incubated for 4 days at 30°C. Following the incubation period measurements of the pH of the individual cultures were made with a Bechman pH meter. The values obtained are shown in (Table XXII).

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It is quite evident from the results presented in the table that those isolates which cause the greatest reduction in the pH of the broth are also the ones which exhibit the greatest antibiotic activity. In only two instances, was this not found to be the case. The pH value obtained for the V48 isolate suggests it is not very active in the production of antibiotic material. Since V48 does produce appreciable amounts of antibiotic it is difficult to explain this result in relation to the high degree of association which is evidenced by the other isolates. The fact that acid was produced by the V48 strain on lactose agar slants, however, suggests that a contaminant may have been present which inhibited the utilization of the carbohydrate by the isolate when it was grown in the broth.

The degree of activity which was shown by the V38 isolate towards various test bacteria in subsequent tests does not support the findings given in Table XXII for this isolate. The property of antibiotic activity which it possessed in some of the earlier studies appears to have been modified. The antibiotic property of V26 appears to have been similarly modified.

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The degree of activity which was shown by the V38 isolate towards various test bacteria in subsequent tests does not support the findings given in Table XXII for this isolate. The recovery of antibiotic activity which it possessed in some of the earlier studies appears to have been significantly modified.

CHROMATOGRAPHY STUDIES

Introduction

Paper chromatography has been successfully used in studies on various antibiotic compounds. Goodall and Levi (32) found the method valuable for testing purified penicillin for traces of other penicillins. Kluener (33) employed a paper chromatographic method for the quantitative estimation of penicillin entities. Chromatography of a broth culture of Penicillium chrysogenum Q-176 showed eight different penicillins to be present in the culture liquor (1). Horne and Pollard (34) in studies on streptomycin have reported a chromatographic method which they describe as an apparently highly selective one for this antibiotic. All the polymyxins have been readily identified following their separation onpaper (35). Polymyxin E was found to be identical in qualitative composition to that of polymyxin A, but its rate of movement on paper was approximately equal to that of polymyxin B. Forsyth (36) studied the antifungal products of several fungi and found the method useful in screening the various fungi for their antibiotic properties.

The outstanding success of the method in determining the chemical nature of antibiotics as well as other organic substances and many inorganic compounds suggested the possibility of its application to antibiotics produced

CHROMATOGRAPHY REVIEW

Introduction

Paper chromatography has been successfully used in studies on various antibiotic components. Locally and Levi (38) found the mobile variable for testing purified penicillin for traces of other penicillins. Gyenes (39) employed a paper chromatographic method for the quantitative estimation of penicillin antities. Horecker (40) of a broth culture of Penicillium notatum-chrysogenum eight different penicillins to be present in the culture liquor (41). Lerne and Tolland (42) in studies on streptomycin have reported a chromatographic method which they describe as an apparently highly selective one for this antibiotic. All the polymyxins have been readily identified following their separation on paper (43). Polymyxin E was found to be identical in qualitative composition to that of polymyxin A, but its rate of movement on paper was approximately equal to that of polymyxin B. Lovegren (44) studied the antifungal properties of several fungi and found the method useful in screening the various fungi for their antibiotic properties.

The outstanding success of the method in determining the chemical nature of antibiotics as well as other organic substances and many inorganic compounds encouraged the possibility of its application to antibiotics occurred

by B. subtilis.

Methods and Results

Broth cultures, broth filtrates, butanol extracts and chloroform extracts of A32 and some of the more active B. subtilis isolates were tested according to the method of Consden et al. (37). Test solutions, of pre-determined antibacterial activity, were spotted (20 ul./spot) on strips of Whatman #1 filter paper and developed with various solvents. The solvent systems employed were:

n-butanol - water;
n-butanol - glacial acetic - water;
n-butanol - ethanol - water;
chloroform - water.

When a solvent had moved the desired distance the paper strips were air-dried and tested for movement of active material. This was done by placing the strips on the surface of a layer of agar which had been seeded with the test organism X-190. The agar layers were contained in 8" x 12" Pyrex-glass trays. The trays to which the strips had been added were placed at 5°C to permit leaching of active material from the strips and at the same time to delay growth of the test bacterium. After a period of 1 hr. at the low temperature, the strips were removed from the trays and then incubated at 30°C for approximately 16 hrs. Following the incubation period, the trays were

by *B. subtilis*.

Methods and Results

Broth cultures, Broth Filtrates, butanol extracts and chloroform extracts of 48 hr. and some of the more active *B. subtilis* isolates were tested according to the method of Dowden *et al.* (31). Test solutions, of pre-determined and bacterial activity, were applied (50 µl./spot) on strips of Whatmen #1 filter paper and developed with various solvents. The solvent systems employed were:

n-butanol - water;

n-butanol - glacial acetic - water;

n-butanol - ethanol - water;

chloroform - water.

When a solvent had moved the desired distance the paper strips were air-dried and tested for movement of active material. This was done by placing the strips on the surface of a layer of agar which had been seeded with the test organism X-190. The agar layers were contained in 8" x 12" Pyrex-glass trays. The trays to which the strips had been added were placed at 5°C to permit leaching of active material from the strips and at the same time to delay growth of the test bacteria. After a period of 1 hr. at the low temperature, the strips were removed from the trays and then incubated at 30°C for approximately 16 hrs. Following the incubation period, the trays were

examined for antibacterial activity.

Paper strips spotted with a mixture of amino acids or a mixture of sugars were developed with the test strips in a few instances as a check on the method. The positions of the amino acids, following development with one of the solvents, were determined by spraying the strips with a .1 percent solution of ninhydrin in n-butanol. The positions of the sugars were determined by spraying the paper with aqueous potassium permanganate (1%) containing sodium carbonate (2%).

None of the solvent systems shown above were found satisfactory when employed for the chromatography of test solutions of A32 and the B. subtilis isolates. None of the strips exhibited any antibacterial activity when tested against X-190. Those systems which contained n-butanol, however, gave good separations of both amino acids and sugars when these substances were developed at the same time as the test strips of the B. subtilis strains. No amino acids or reducible carbohydrates were found to be present when the strips spotted with the antibiotic solutions were sprayed with ninhydrin or potassium permanganate solutions respectively.

Other attempts to obtain a migration of active material along the paper were also made. The methods used for the chromatography of streptomycin (34) and penicillin (32) were found ineffective.

Following these trials, a filter-paper strip, which

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None of the solvent systems chosen above were found satisfactory when applied for the chromatography of test solutions of A32 and the D. multilia isolates. None of the strips sampled any antibacterial activity when tested against X-120. Those systems which contained n-butanol, however, gave good separations of both amino acids and sugars when these substances were developed at the same time as the test strips of the D. multilia strains. No amino acids or reducible carbohydrates were found to be present when the strips sprayed with the antibiotic solutions were sprayed with ninhydrin or potassium permanganate solutions respectively.

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Following these trials, a filter-paper strip, which

had not been spotted with any of the test solutions, was suspended in a boiling tube with one end of the strip immersed in a broth filtrate of A32. Movement of the filtrate solution up the strip was apparent. When the liquid had moved the length of the paper, the paper was then air-dried and tested for antibacterial activity against X-190. Only that portion of the strip which had been immersed in the broth filtrate solution showed any activity when tested against the test bacterium. Paper strips, used to test butanol extracts of A32 by the same procedure, exhibited antibacterial activity throughout their length.

The active material of the A32 strain during the process of partial purification apparently undergoes a loss in water solubility. This became apparent on testing distilled water to which a small amount of A32 solid material was added, for activity against a test bacterium. No activity was exhibited by the water preparation. Heating the suspension to approximately 95°C for 5 minutes did not appear to increase the solubility of the active material in water.

Datta et al. (38) found paper which had been impregnated with aluminum sulfate useful for the separation of vitamin A and its derivatives. They emphasized the possibility of extending the method to the separation of large water-insoluble organic molecules. When the method was applied to chloroform extracts of a few of the B. subtilis strains, movement of the active material was not obtained. Antibacterial activity was exhibited only by

had not been exceeded with any of the test solutions, was suspended in a boiling tube with one end of the strip immersed in a broth filtrate of 1:32. Movement in the filtrate solution up the strip was apparent. When the liquid had moved the length of the paper, the paper was then air-dried and tested for antimicrobial activity against *P.-ISO*. Only that portion of the strip which had been immersed in the broth filtrate solution showed any activity when tested against the test bacterium. Paper strips, used to test natural extracts of ASS by the same procedure, exhibited antimicrobial activity for months (Beir Laugh).

The active material of the ASS which during the process of partial purification spontaneously undergoes a loss in water solubility. This became apparent on testing distilled water to which a small amount of ASS solid material was added, for activity against a test bacterium. No activity was exhibited by the water preparation. Heating the suspension to approximately 65°C for 5 minutes did not appear to increase the solubility of the active material in water.

Deeba *et al.* (58) found paper which had been impregnated with aluminum sulfate useful for the separation of vitamin A and its derivatives. They experimented the possibility of extending the method to the separation of large water-insoluble organic molecules. When the method was applied to chloroform extracts of a few of the *E. antillis* strains, movement of the active material was not obtained. Antibacterial activity was confirmed only by

those areas where the chloroform solutions had been spotted on the paper. Irrigation of the paper with chloroform and benzene did not result in any movement of active material along the strips.

The most promising method employed in the chromatography studies was that of Kritchevsky and Tiselius (39). They found that paper which had been drawn through a 5 percent solution of silicone in cyclohexane proved to be a satisfactory support for the separation of substances such as the steroids which have limited solubility in water. In the studies reported here, the following procedure was employed.

Strips of Whatman #1 filter-paper measuring 10 x 30 cm. were drawn through a 5 percent solution of Dow Corning silicone #1107 in cyclohexane. The paper was rendered hydrophobic by this treatment and readily adsorbed vapors of chloroform. After the papers had been treated with silicone, they were blotted between absorbent papers and dried at 105°C for 1 hr. Thirty ul. of different chloroform extracts were spotted at 2 cm. intervals along a line 7 cm. from one end of the paper.

The solvent system employed consisted of 6 vol. of distilled water, 10 vol. of absolute ethanol and 10 vol. of chloroform. These were shaken in a separatory funnel. After a period of 1 hr. the non-polar layer was added to the bottom of the chromatography chamber. The paper on which the test solutions had been spotted was then transferred to the chamber and suspended from a glass trough.

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The most promising method employed in the chromatographic studies was that of Lieberman and Liebling (59). They found that paper which had been dipped through a 6 percent solution of silicone in cyclohexane proved to be a satisfactory support for the separation of substances soon as the steroids which have limited solubility in water. In the studies reported here, the following procedure was employed.

Strips of Whatman #1 filter-paper measuring 10 x 60 cm. were drawn through a 6 percent solution of Dow Corning silicone #200 in cyclohexane. The paper was repeatedly dipped by this treatment and readily adsorbed vapors of chloroform. After the papers had been treated with silicone, they were blotted between absorbent papers and dried at 105°C for 1 hr. Thirty ml. of different chloroform extracts were spotted at 2 cm. intervals along a line 7 cm. from one end of the paper.

The solvent system employed consisted of 8 vol. of distilled water, 10 vol. of absolute ethanol and 10 vol. of chloroform. These were shaken in a separatory funnel. After a period of 1 hr. the non-polar layer was added to the bottom of the chromatography chamber. The paper on which the test solutions had been spotted was then introduced by the chamber and suspended from a glass rod.

The chamber was left standing for 1 hr. to equilibrate both the chamber and the paper with respect to the non-polar or stationary phase. The polar or mobile phase was then added to the trough and the paper was irrigated with this phase for 22 hr.

After the solvent had moved the desired distance along the strip, the paper was removed from the chamber and air-dried. It was then tested for movement of active material against the test organism X-190.

When the trays were examined following an incubation period, no movement of antibiotic material was apparent. However, inhibition zones were present which corresponded to the positions on the paper at which the chloroform extracts were spotted.

In a second trial, 105 ul. of chloroform extract were spotted on the paper. The paper was developed as in the foregoing test, but the volumes of the different solvents employed were 4 vol. of water, 10 vol. of absolute ethanol and 10 vol. of chloroform. When the papers were tested for antibacterial activity, inhibition zones corresponding to the positions at which the test solutions had been spotted were apparent. In addition, a large zone of inhibition was present which corresponded to the position on the paper marking the solvent front.

In a further trial, the papers were again spotted with 105 ul. of test solution and then developed with the mobile phase of a solvent system consisting of 6 vol. of water, 10 vol. of absolute ethanol and 10 vol. of chloroform.

The chamber was left standing for 1 hr. to equilibrate both the chamber and the paper with reference to the non-polar or stationary phase. The polar or mobile phase was then added to the trough and the paper was irrigated with this phase for 20 hr.

After the solvent had moved the desired distance along the strip, the paper was removed from the chamber and air-dried. It was then tested for movement of active material against the best organism X-100.

When the strips were examined following an incubation period, no movement of detectable material was apparent. However, inhibition zones were present which corresponded to the positions on the paper at which the chloroform extracts were spotted.

In a second trial, 100 ml. of chloroform extract were spotted on the paper. The paper was developed as in the foregoing test, but the volumes of the different solvents employed were 4 vol. of water, 10 vol. of absolute ethanol and 10 vol. of chloroform. When the papers were tested for antibacterial activity, inhibition zones corresponding to the positions at which the test solutions had been spotted were apparent. In addition, a large zone of inhibition was present which corresponded to the position on the paper reaching the solvent front.

In a further trial, the paper was again spotted with 100 ml. of test solution and then developed with the mobile phase of a solvent system consisting of 4 vol. of water, 10 vol. of absolute ethanol and 10 vol. of chloroform.

These conditions resulted in a movement of active material along the strip. In this trial, however, the inhibition zones did not coincide with the position on the paper marked by the solvent front. The distance the active materials had moved from the starting line relative to the distance the solvent had moved from the same position was calculated and expressed as a ratio. The values obtained for the various B. subtilis strains tested were A32 - .850, V3 - .954, V10 - .868 and V16 - .909. These values represent the position of only those zones of inhibition which were clearly defined. Other zones of inhibition which were less distinct were also apparent.

Discussion

Paper chromatography has been extensively used for the separation and identification of many compounds. Many solvent systems have been reported. However, in almost all of these, water has been used as the stationary phase. The use of such systems for substances which are relatively insoluble in water limits the use of paper chromatography for these compounds.

In the present studies, those systems in which water was employed as the stationary phase failed to give satisfactory results when applied to the active material of the B. subtilis strains tested. As A32 solid material is apparently either insoluble or only very slightly soluble

Discussion

in water, this may be one explanation for such negative results. Asparagine broth medium contains the mineral salts dipotassium phosphate, magnesium sulfate and potassium chloride. The fact that the active material of an A32 broth filtrate did not move along the paper when strips were suspended in it, suggests that the antibiotic is immediately "salted out" by this treatment. In the earlier studies, only small quantities of the test solutions were applied to the papers. Since filter-paper can effectively adsorb antibiotics produced by strains of B. subtilis, this may have been an additional reason for the lack of movement of the active materials in the earlier studies.

In the studies employing filter-paper, impregnated with aluminum sulfate, inhibition zones were obtained only at those positions on the paper to which the chloroform extracts had been applied. These results suggest that the treatment of the paper with aluminum sulfate caused the active material to be strongly adsorbed to it and therefore irrigation with chloroform and benzene was ineffective.

Kritchevsky et al. (39) found that substances having only limited solubility in water could be readily separated by reversing the phases of a two-phase solvent system. This was effected by treating the paper with silicone. Following such a treatment the non-polar phase, which is adsorbed by the paper, acts as the stationary phase; the water, or polar-phase, acts as the mobile phase. When this procedure was employed for test solutions of B. subtilis strains, the movement of antibiotic material was found to

depend on both the quantity of test solution applied to the paper and the volume of water included in the solvent system. Although some active material was retained at those positions at which the test solution were applied to the paper, the degree of adsorption of antibiotic to the paper does not appear to be very great. When larger quantities of the test solutions were applied, movement of active material was apparent. The rate of movement of the antibiotics in relation to the rate at which the solvent moves apparently depends on the volume of water which is included in the solvent system. By increasing the volume of water from 4 volumes to 6 volumes, the rate at which the antibiotics moved was noticeably decreased. On the basis of the results obtained in the present studies, reversed phase paper partition chromatography should prove to be a valuable method for determining the chemical nature of those antibiotics produced by B. subtilis which have limited solubility in water.

GENERAL CONCLUSIONS AND DISCUSSION

During the past few years much interest has been shown in the bacterium B. subtilis. This interest has arisen in part from the many reports describing antibiotics produced by this species. The present studies designed to ascertain the variability in antibiotic production of local strains of this species, have demonstrated among them, vary-

depend on both the quantity of heat radiation applied to the paper and the volume of water included in the mixture system. Although some active material was removed at three positions at which the test solution were applied to the paper, the degree of absorption of antibiotic to the paper does not appear to be very great. When larger quantities of the test solutions were applied, movement of active material was apparent. The rate of movement of the antibiotics in relation to the rate at which the solvent moves apparently depends on the volume of water which is included in the solvent system. By increasing the volume of water from 4 volumes to 8 volumes, the rate at which the antibiotics moved was noticeably decreased. On the basis of the results obtained in the present studies, reversed phase paper partition chromatography should prove to be a valuable method for determining the chemical nature of these antibiotics produced by B. subtilis which have limited solubility in water.

GENERAL CONSIDERATIONS AND DISCUSSION

During the past few years much interest has been shown in the bacterium B. subtilis. This increased use arises in part from the many reports describing antibiotics produced by this species. The present studies designed to ascertain the variability in antibiotic production of local strains of this species, have demonstrated among them, vari-

ing degrees of antibacterial activity ranging from inactivity to strong activity towards the test organisms used. Approximately 50 percent of the strains tested were found to produce appreciable amounts of antibiotic material.

The A32 strain was shown to be a much more active strain than many of the isolates tested and to be equal to several others in the degree of antibiotic activity which it exhibits. If media and test conditions other than those which were employed, had been used in the studies, different results might have been obtained. The conditions used in the tests were those found by Goodman (18) to support maximum antibiotic production by A32. These conditions, however, may not have been optimum for maximum antibiotic production by some of the isolates. Foster and Woodruff (9) have reported that the bacillin strain of B. subtilis exhibits two types of activity depending on the culture medium employed. Hirschorn et al. (15) reported that the medium used for the production of subtenolin greatly influences the yields of this antibiotic. A medium containing d l-alanine gave the highest yields, whereas, media containing peptones and complex organic enrichments were found to be very poor. Subtenolin production was slightly stimulated by the presence of copper and strongly stimulated by the presence of manganese. Subtilin production is similarly affected by the presence of the latter element (7). In the present studies only asparagine broth medium was employed. If other media had

ing degrees of antibacterial activity ranging from inactivity to strong activity towards the test organisms used. Approximately 50 percent of the strains tested were found to produce appreciable amounts of antibiotic material.

The ASP strain was shown to be a much more active strain than many of the isolates tested and is equal to several others in the degree of antibiotic activity which it exhibits. If media and test conditions other than those which were employed, had been used in the studies, different results might have been obtained. The conditions used in the tests were those found by Goodman (18) to support maximum antibiotic production by ASP. These conditions, however, may not have been optimum for maximum antibiotic production by some of the isolates. Porter and Woodruff (9) have reported that the bacillin strain of B. subtilis exhibits two types of activity depending on the culture medium employed. Tirpsoro et al. (15) reported that the medium used for the production of subtenolin greatly influences the yields of this antibiotic. A medium containing d l-alanine gave the highest yields, whereas, media containing peptones and complex organic substances were found to be very poor. Subtenolin production was slightly stimulated by the presence of copper and strongly stimulated by the presence of manganese. Bacillin production is similarly affected by the presence of the latter element (7). In the present studies only asparagine broth medium was employed. It other media had

been used some of the weakly active strains might have exhibited greater degrees of antibacterial activity.

When the antibacterial spectra of the isolates are compared with the spectrum of A32, pronounced differences, as determined by various methods, are apparent. These differences are believed to be related to differences in the types of antibiotics produced. In Table XXIII the isolates which exhibited such differences, as determined by the various studies indicated in the table, are listed. The antibiotics produced by some of the isolates shown in the table may prove valuable not only for experimental purposes but also in the control of plant and animal diseases.

Studies on the polymyxins have demonstrated that the differences exhibited by the individual antibiotics are not absolute (29, 40). They are, important, however, and are related to differences in the chemical nature of the compounds. This has been confirmed by various chemical and biological studies, but more particularly by the use of paper chromatography (35). By this procedure the amino acids threonine and α - γ - diamino butyric acid were found by Jones (35) to enter into the composition of all the polymyxins studies. However, the presence or absence of the amino acids D-leucine, phenylalanine and serine varied with the antibiotic. These differences and the rate of movement of the intact antibiotic on paper have been used to classify the polymyxins.

TABLE XXIII

Isolates whose antibiotic material differs in type from that of B. subtilis A32

Spot-inoculation studies	Broth filtrate studies		Broth culture studies	Comparative studies on broth cultures and broth filtrates	Extraction studies
	Berkefeld filtered	Seitz filtered			
V9	V2	V10	V9	V3	V10
V33	V3	V13	V10	V12	V16
V34	V8	V16	V30	V16	V35
	V10	V20	V48	V20	
	V11	V25		V25	
				V30	
				V48	

TABLE XXIII

Isolates Whose Morphologic Appearance Differs in Type from that Described in Subtitle ASC

Isolates	Coccus-incontrasting colonies	Coccus filtrate studies		Broth culture studies	Decomposition of Dorset egg medium and extracellular slime
		Bentonite filtered	Soils bentonite filtered		
49	45	420	420	43	420
433	433	433	433	425	424
434	434	424	424	424	434
		420	420	420	
		434	434	434	
				420	
				448	

A situation similar to that found to exist with the polymyxins may become apparent when more data on the chemical nature of the antibiotics of B. subtilis are obtained. The differences shown for some of the isolates, while not absolute, are marked differences in several instances. It may be that the antibiotics produced by some of the isolates differ from that of the A32 strain in either the size of the molecule or the presence of various constituent groups. As only one broth medium was employed in the studies this may have resulted in the production of a substance common to each strain with possibly other active materials produced in varying amounts.

High antibiotic activity was found to be closely associated with lactose fermenting ability in the strains of B. subtilis studied. This close association between these two properties suggests that the fermentation of lactose may prove to be a valuable criterion for the isolation of new antibiotic producing strains. Such a criterion would be particularly useful in screening large numbers of B. subtilis isolates for their antibiotic properties.

Jansen and Hirschmann (7), as previously described, have shown cultures high in subtilin activity to give an intense blue color on the addition to the medium of a few drops of alcoholic ferric chloride. This was not found to be a property of any of the isolates tested in the present studies. If a medium favorable to the production of subtilin had been employed, some of the isolates might

have given a positive reaction to the test. Goodman (18), however, on comparing the antibiotic properties of A32 and a subtilin producing strain found the subtilin strain to give a positive ferric chloride test even when grown in a medium supporting maximum production of A32 antibiotic. This suggests that the isolates differ in their antibiotic properties from subtilin producing strains of B. subtilis.

The reversed phase method of paper partition chromatography shows considerable promise as a procedure for the separation of the antibiotics produced by B. subtilis isolates. If it should prove to be a successful method it will greatly facilitate the classification of antibiotics produced by new antibiotically active strains. Other procedures employed in paper chromatography may prove useful in studies on hydrolyzed material. However, unless the antibiotic is in a pure form it would be difficult to separate the impurities from the actual constituents of the active materials. Such impurities may be the result of growth products of the isolate which are antibiotically inactive.

The marked variability exhibited by the species B. subtilis may prove to be a valuable property in future fundamental research on antibiotics.

SUMMARY

1. This investigation was undertaken to study the variability in antibiotic production of local strains of Bacillus subtilis. A strain of this bacterium, referred to as A32, was used as a standard with which the antibiotic properties of the isolates obtained in the present study were compared.

2. Soil and wheat seed were tested particularly for organisms resembling A32 in growth characteristics. Various other materials were examined for the presence of this and other strains of B. subtilis.

3. Isolates resembling A32 were not found to be commonly present on or in the various materials tested. This was particularly true of soil and seed.

4. The A32 strain could not be recovered from non-sterile soil to which it had been added.

5. A total of 54 isolates were obtained from various sources and tentatively identified as strains of B. subtilis. The antibiotic properties of 51 of these were studied.

6. Marked variability in antibiotic activity as determined by spot-inoculation tests was exhibited by the isolates towards strains of Xanthomonas translucens. Fifty-four percent of the isolates showed appreciable amounts of antibiotic activity towards another test bacterium X-190.

7. Broth cultures of some of the isolates after filtration through Berkefeld and Seitz bacterial filters differed

in the types of antibiotic present in them.

8. In studies employing non-filtered broth cultures of B. subtilis strains, a wide range of antibacterial activity was exhibited. Fifty-two percent of the isolates tested were shown to produce appreciable amounts of antibiotic activity against the test organism X-190.

9. Both Berkefeld and Seitz bacterial filters were found to remove antibiotic material from broth cultures of the isolates.

10. When the antibacterial activities of broth cultures and broth filtrates were compared, results were obtained which indicate that some of the isolates produce active material which differs from the type of antibiotic produced by A32.

11. Extraction of broth cultures with n-butanol effectively removed antibiotic material produced by the more active isolates. When butanol extracts were distilled in vacuo over water, extraction of the water layers with chloroform effectively removed the active material.

12. Strains of B. subtilis exhibiting the greatest antibiotic activity were found to have a rough type of growth. Some of the rough strains, however, were inactive. Isolates whose growth was smooth were either only weakly active or inactive.

13. Isolates which produced a pink pigment when grown on potato slices were only weakly active in the production of antibiotic material.

14. The alcoholic ferric chloride test for the produc-

tion of subtilin was negative for all strains.

15. High antibiotic activity was found to be closely associated with the lactose fermenting ability of the isolates.

16. Paper partition chromatography gave negative results when solvent systems in which water was employed as the stationary phase were tested.

17. Reversed phase paper partition chromatography was found to be a promising method which may prove valuable in the separation and classification of antibiotic materials produced by B. subtilis strains having similar antibiotic properties to those used in the present study.

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LITERATURE CITED

1. WINSTEN, W.A. and SPARK, A.H. Penicillin types produced by Penicillium chrysogenum Q-176. Science 106:192-193. 1947.

2. SCHATZ, A., BUGIE, E. and WAKSMAN, S. A. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc. Soc. Exp. Biol. and Med. 55:66-69. 1944.

3. WAKSMAN, S.A., SCHATZ, A. and REILLY, H.C. Metabolism and the chemical nature of Streptomyces griseus. J. Bact. 51:753-759. 1946.

4. LEACH, B.E., FORD, J.H. and WHIFFEN, A.J. Actidione, an antibiotic from Streptomyces griseus. J. Amer. Chem. Soc. 69:474. 1947.

5. WHIFFEN, A.J., BOHONOS, N. and EMERSON, R.L. The production of an antibiotic substance by Streptomyces griseus. J. Bact. 52:610-611. 1946.

6. PORTER, J.H., BRASCHARD, R., KRUPKA, G., LITTLE, P. and ZELLAT, J.S. Isolation and production of polymyxin. Ann. N.Y. Acad, Sci. 51:853-1000. 1949.

7. JANSEN, E.F., and HIRSCHMANN, DORIS J. Subtilin-- an antibacterial product of Bacillus subtilis. Culturing conditions and properties. Arch. Biochem. 4:297-309. 1944

8. JOHNSON, BABBINA, A., ANKER, N., and MELENY, F.L. Bacitracin. A new antibiotic product of the Bacillus subtilis group. Science 102:376. 1945.

9. FOSTER, J.W., and WOODRUFF, H. H. Bacillin, a new antibiotic from a soil isolate of Bacillus subtilis. J. Bact. 51:363-369. 1946

10. VALLE, M. Étude du pouvoir bacteriolytique du Bacillus subtilis. Lysine et analysine. Biol. Abst. 20:12968, 1412. 1946.

11. OLIVIER, H.R., de SAINT-RAT, L., BONNET-MAURY, P. and BLACHON, E. Etude de l'activité d'un extrait de corps microbiens de Bacille subtil sur le Straphylocoque. Biol. Abst. 20:17182, 1900. 1946.

12. RAMON, G. and RICHOU, R. De L'influence sur les propriétés antibiotiques et antibiotiques des filtrats du B. subtilis, de la chaleur et de l'aldehyde formique agissant simultanement. Biol. Abst. 20:15729, 1682. 1946.

13. NEWTON, G.F. Antibiotics from a strain of Bacillus subtilis: Bacilipin A and B and Bacilysin. Brit. J. Exp. Path. 30:306-319. 1950

14. JOHNSON, E.A. and BURDON, K. L. Eumycin - a new antibiotic active against pathogenic fungi and the higher bacteria, including bacilli of tuberculosis and diptheria. J. Bact. 51:591. 1946.

15. HIRSCHORN, H.N., BUCCA, M.A. and THAYER, J.D. Subtenolin. An antibiotic from Bacillus subtilis. 1. Bacteriologic properties. Proc. Soc. Exp. Biol. 67:429. 1948.

16. CALLOW, R.K. and D'ARCY HART, P. Antibiotic material from Bacillus licheniformis active against species of Mycobacteria. Nature 157:334-335. 1946.

17. JACKSON, A.W. Private communication. 1950.

18. GOODMAN, J.J. On the value of a substance of bacterial origin in the treatment of seed. Thesis. Univ. of Alberta. 1947.

19. GORDON, D. Private communication. 1950

20. SMITH, N.R., GORDON, RUTH E. and CLARK, F.E., Aerobic mesophilic sporeforming bacteria. U. S. Dep. Agr. Misc. Pub. 559:pp. 112. 1946.

21. GIBSON, T. and TOPPING, L.E. Further studies of the aerobic sporeforming bacilli. Proc. Soc. Bact. Abst. 43-44. 1938.

22. GIBSON, T. A study of Bacillus subtilis and related organisms. J. Dairy Res. 13:248-260. 1944.

23. FRED, E.B. and WAKSMAN, S.A. Laboratory manual of general microbiology. McGraw-Hill Book Co., Inc. New York. 1928.

24. Manual of Methods for Pure Culture Study of Bacteria. Soc. of Amer. Bacteriologists. Leaflet V. 1944.

25. ROBBINS, W.J., KAVANAGH, F. and HERVEY, ANNETTE Production of antibiotic substances by basidiomycetes. Ann. N.Y. Acad. Sci. 48:67-73. 1946.

26. WAKSMAN, S.A., SCHATZ, A. and REYNOLDS, D.M. Production of antibiotic substances by actinomycetes. Ann. N.Y. Acad. Sci. 48:73-87. 1948.

27. VINCENT, J.G. and VINCENT, H.W. Filter-paper disc modification of the Oxford cup penicillin determination. Proc. Soc. Exp. Biol. and Med. 55:162-164. 1944.

28. STANSLY, P.G. and SCHLOSSER, M.E. Studies on polymyxin: an agar diffusion method of assay. J. Bact. 54:585-597. 1947.

29. BENEDICT, R.G. and STODOLA, F.H. Effect of various factors on the production of polymyxin. Ann. N.Y. Acad. Sci. 51:857-866. 1949.

30. RAKE, G. and RICHARDSON, A.P. Pharmacology in penicillin. Ann. N.Y. Acad. Sci. 48:143-175. 1946.

31. RAPER, K.B. The development of improved penicillin-producing molds. Ann. N.Y. Acad. Sci. 48:41-57. 1946.

32. GOODALL, R.R. and LEVI, A.A. A microchromatographic method for the detection and approximate determination of the different penicillins in a mixture. Nature. 158:675. 1946.

33. KLUENER, R.G. A paper chromatographic method for the quantitative estimation of penicillin entities. J. Bact. 57:101-109. 1949.

34. HORNE, R.E. and POLLARD, A.L. The identification of streptomycin on paper strip chromatograms. J. Bact. 55:231-234. 1948.

35. JONES, T.S.G. Chemical evidence for the multiplicity of the antibiotics produced by Bacillus polymyxa. Ann. N.Y. Acad. Sci. 51:909-917. 1949.

36. FORSYTH, W.G.C. The identification of antifungal substances (produced by fungi) by paper chromatography. Fifth Inter. Cong. Microbiology. 1950.

37. CONSDEN, P., GORDON, A.H. and MARTIN, A.J.P. Quantitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. 38:224-232. 1944.

38. DATTA, S.P., OVERELL, B.G. and STACK-DUNNE, M. Chromatography on alumina-impregnated filter-paper. Nature 164:674. 1949.

39. KRICHEVSKY, T.H. and TISELIUS, A. Reversed phase partition chromatography of steroids on silicone-treated paper. Science 114:299. 1951.

40. WHITE, H.J., ALVERSON, C.M., BAKER, M.J. and JACKSON, E.R. Comparative biological studies of polymyxin and aerosporin. Ann. N.Y. Acad. Sci. 51:879-891. 1949.

APPENDIX "A"

The identity of some of the test organisms employed in the present studies is uncertain. They have, therefore, been given numerical designations which are used throughout the text. The test bacteria so described are as follows:

X-190 *Xanthomonas translucens f. sp. hordei-avenae*

X-191 *Erwinia amylovora*

X-192 *Bacillus megatherium*

X-193 *Bacterium atrofaciens*

X-194 *Bacterium agropyrii*

X-195 *Bacillus cereus*

X-196 *Bacillus subtilis* 44

X-197 *Bacillus subtilis* 43

X-198 *Bacillus subtilis* 33

APPENDIX "A"

The identity of some of the test organisms employed in the present studies is uncertain. They have, therefore, been given numerical designations with are used throughout the text. The test bacteria so designated are as follows:

X-126 *Xanthomonas translucens* f. sp. *hordei-vaccae*

X-127 *Erwinia amylovora*

X-128 *Bacillus megatherium*

X-129 *Bacterium atrofaciens*

X-12A *Bacterium aurofaciens*

X-12B *Bacillus cereus*

X-12C *Bacillus subtilis* #4

X-12D *Bacillus subtilis* #3

X-12E *Bacillus subtilis* #2